Hepatitis A virus in relation to contamination of factor VIII

Thesis

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HEPATITIS A VIRUS IN RELATION TO CONTAMINATION OF FACTOR VIII

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This thesis is dedicated
to
my husband
and
Parents

CONTENTS

ABSTRACT

Outbreaks of hepatitis A amongst hemophiliacs receiving solvent detergent treated factor VIII in Europe and the USA have recently caused concern to blood product manufacturers and control agencies. In this study, the use of terminal dry heat treatment to inactivate HAV (strains HM175A.2 and HM175/18f) in spiked factor VIII was assessed using tissue culture based HAV assays, including a novel cytopathic microtitre plate assay, and a quantitative RT-PCR assay. The infectivity of HAV was quickly destroyed by heating to 80 °C. However, it was not until samples were heated to 100 °C for 8 hr that PCR positivity was lost. The use of nucleic acid amplification techniques in blood transfusion virology is increasing due to the lack of suitable cell culture assays for viruses such as HCV and parvovirus B19. This study shows that ability to detect viral genome in blood products does not necessarily imply the presence of infectious virus after the use of virucidal treatments. The relationship between PCR positivity and infectivity of HAV was further assessed in a time course study of cytopathic HAV HM175A.2 replication in BS-C-1 cells. Titres of released HAV RNA were similar to that of infectious virus with a ratio of 2 genome equivalents per infectious unit, whereas 19 genome equivalents per infectious unit were found associated with the cell. The infectious titre of contaminating HAV in blood products prior to viral inactivation was similar to that of HAV RNA. Comparison of infectious titres with genome copies of HAV during the time course demonstrated that little viral RNA was available for replication in the early stages of infection due to efficient encapsidation. The uptake and replication of the less cytopathic strain, HM175/18f, was slower than that of HM175A.2, however similar RNA titres were reached. The ability of HAV to cause cytopathology may be related to rate of replication. The attenuation of HM175A.2 and HM175/18f was studied in a tamarin model. HAV HM175/18f showed a degree of attenuation compared to wild type HM175 and HM175A.2 appeared to be completely attenuated. There was no difference in time of seroconversion between strains. HAV HM175A.2 may prove to be a suitable vaccine strain.

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LIST OF FIGURES

LIST OF TABLES

Chapter 1

INTRODUCTION

1.1 HISTORY

Symptoms of liver disease were documented as early as 5th century BC in the Babylonian Talmud which describes the common appearance of jaundice in the population. In Greek and Roman literature of the same era the epidemic nature of some liver conditions was noted. Hippocrates (460-375 BC) classified an epidemic form of jaundice as the 'fourth kind of jaundice', however, contemporary understanding of the nature of epidemic diseases was based on the four humours which included yellow bile as a common source of fever. In the 8th century AD, in a letter to St. Boniface, Archbishop of Mainz, Pope Zacharias encouraged the separation of jaundiced individuals from others due to the contagious nature of the disease.

Sporadic outbreaks of epidemic jaundice were not commonly noted until the 18th century when written accounts reported several epidemics which occurred throughout Europe (reviewed by Achernknecht, 1968). Epidemic jaundice has been a particular problem amongst the military during wars from the Middle Ages and therefore became known as 'campaign jaundice'. Extremely large epidemics of hepatitis were recorded during both the First and Second world wars. This campaign jaundice was usually characterised by transient fever, malaise, headache, anorexia, nausea, abdominal discomfort and eventually, the appearance of jaundice (Macpherson *et al.*, 1921).

The common view during the 19th century was that jaundice was due to swelling and obstruction of the hepatic duct or the common bile duct, rather than disease of the liver itself, giving rise to the terms obstructive or catarrhal jaundice (MacCallum, 1972). However, several clinicians at that time suspected the liver to be the site of primary pathology and that infection had a role in the aetiology of jaundice. In 1912, Cockayne described epidemic jaundice as infectious hepatitis, postulating that the pathogen reaches the liver via the blood being transmitted by the aerosol route. A popular belief at this

time, as expressed by McDonald (1908) was that an infectious agent caused necrosis of a previously damaged liver. Both Cockayne and McDonald proposed that the pathogen may be a virus due to the epidemic nature of the disease and the inability to isolate a bacterial agent. This view soon gained support (Martin, 1915; Stokes *et al.*, 1920; Findlay *et al.*, 1931). In a report on outbreaks of epidemic hepatitis in the USA between 1918 and 1922, Blumer (1923) established the occurrence of infectious hepatitis, now known as infectious hepatitis type A. It was not until during and after the Second World War when human volunteer studies were performed that the viral aetiology of hepatitis was finally established (reviewed by MacCallum *et al.*, 1951; Havens, 1947; 1963).

The earliest report of jaundice being transmitted via serum was in 1883 amongst shipyard workers of Bremen (reviewed by MacCallum, 1947). After vaccination against smallpox with glycerated human lymph, 191 of 1289 vaccinated employees became jaundiced within 6 months. It was discovered that workers inoculated with a different batch of lymph did not develop symptoms of hepatitis. Early in the 20th century, the use of long needles in Salvarsen therapy administered by venepuncture in the treatment of syphilis brought about reports of outbreaks of jaundice occurring at many venereal disease clinics (Bigger, 1948). Due to the high numbers of patients at these clinics, there was not time to sterilise needles between each patient. Outbreaks of jaundice were also attributed to the use of shared needles in administration of insulin to diabetics and gold injections to sufferers of rheumatoid arthritis (MacCallum, 1943). After an outbreak of 41 cases of jaundice and subsequently 8 deaths following immunisation against measles using a convalescent human serum, a report by MacNalty for the Ministry of Health (1938) classified this type of jaundice as serum hepatitis. The spread of this disease after administration of other human serum based immunotherapies and on transfusion of serum, plasma or whole blood became a recognised risk.

Epidemiological observations led to the classification of infectious hepatitis and serum hepatitis into 2 distinct groups based mainly on length of incubation period and route of transmission. In 1947, MacCallum proposed the names hepatitis type A for infectious hepatitis and hepatitis type B for serum hepatitis which were adopted in 1973 by the Scientific Group on Viral Hepatitis of the World Health Organisation.

Between 1956 and 1973 studies were carried out by Krugman and co-workers (Krugman *et al.*, 1967; Krugman and Giles, 1970) at an institution for the mentally handicapped in New York which confirmed that there were 2 distinct groups of viral hepatitis. Epidemics of hepatitis were frequent at this institution and the majority of children acquired the illness within the first 6-12 months after admission. Using subjects at the school, it was shown that by about 4 weeks after inoculation with serum MS-1, children became ill with a type of hepatitis that was highly transmissible by contact, typical of hepatitis A. Children inoculated with another serum, MS-2 did not develop symptoms as quickly (Incubation period > 3 months) and did not generally pass on the virus by contact. The immunoreactivity of the MS-1 strain was impaired by heating to 98 °C for 1 minutes whereas that of MS-2 was unaffected (Krugman *et al.*, 1970). The MS-1 agent was subsequently classified as a type A hepatitis virus and MS-2 as type B hepatitis virus. When adult volunteers were inoculated with MS-1 agent, they developed the same symptoms as the inoculated children (Boggs *et al.*, 1970). The pathogen was extracted from the stools of these patients and identified as a virus-like agent by immune electron microscopy by Feinstone *et al.*(1973).

After many frustrated attempts by several groups, Provost and Hilleman (1979) demonstrated infection of primary marmoset liver cell cultures and foetal rhesus monkey kidney cells with hepatitis A virus strain CR326 which had been serially passaged in

marmosets (Mascoli *et al.*, 1973). Despite the long history of HAV infection, acquisition of knowledge about the biology of the virus has been extremely difficult. The slow growth and inability to produce high titres of HAV in tissue culture have particularly hindered attempts to study the virus.

1.2 PATHOGENESIS

Hepatitis A virus is transmitted via oral and percutaneous routes, usually by ingestion of food or drink contaminated with faeces containing the virus. The main site of viral replication and pathogenesis is the liver, however, the early events of infection in which the virus travels via the gastrointestinal tract to the liver remained little understood. Attempts to demonstrate primary extrahepatic replication of HAV have been inconclusive. Detection of HAV in the saliva of both humans (Purcell *et al.*, 1984) and experimentally inoculated chimpanzees (Cohen *et al.*, 1989) suggested that the virus replicates in the salivary glands however replication at this site has not been shown. Lemon (1985) suggested that the HAV may have been detected in the saliva due to contamination with viraemic blood.

The incubation period for HAV in humans is usually between 15 - 50 days with a mean of 30 days. A transient viraemic phase lasting up to 2 weeks occurs before the onset of disease symptoms. Purcell and co-workers (1984) found the maximum titre of HAV detected in peripheral blood to be 10^5 chimpanzee infectious units per ml. Faecal shedding also commences towards the end of the incubation period and is at a peak immediately prior to the onset of early disease symptoms. In the above study, viral titres of up to 10^9 chimpanzees infectious units per gram were detected in human stool samples. By the time jaundice is apparent, HAV is usually no longer detectable in the faeces.

The pathological changes seen in HAV infection are similar to those caused by HBV (Dienstag *et al.*, 1975; Ishak, 1976). Acute hepatitis is characterised by 'spotty liver necrosis' and intralobular and portal lymphohistiocytic (involving infiltration by lymphocytes and macrophages) inflammation. Perivenular lytic necrosis and a randomly distributed eosinophilic type of necrosis are also observed. A picture of lobular disarray is formed by the coexistence of degeneration and regeneration of hepatocytes. Degeneration of these cells is distinguished by the appearance of swelling, indistinct plasma membranes, enlarged nuclei and featureless cytoplasm. Cellular debris and necrotic cells migrate into the sinusoids via the spaces of Disse where they are digested by the phagocytic Kupffer cells. As the Kupffer cells in the sinusoids and macrophages in the portal ducts become activated, both the size and number of cells in these structures increase. Enlargement of the portal tracts also results from oedema and cellular infiltration involving lymphocytes and, to a lesser extent, neutrophils and eosinophils. As the patient recovers, regeneration is characterised by mitosis and multinucleated cells. Inflammatory cells begin to recede and the hepatic tissue is usually restored within 8-12 weeks.

The specific events that occur upon infection of the liver with HAV and lead to inflammation and necrosis have not yet been clarified. HAV antigen has been demonstrated in up to 100 % of hepatocytes and also in Kupffer cells of experimentally inoculated non-human primates. It was thought that the virus may be directly cytotoxic to hepatocytes, thereby facilitating the release of virions, however, maximal virus shedding occurs prior to the appearance of widespread cytopathology in the liver (Keenan *at al.*, 1984; Karayiannis *et al.*, 1986). When cultivated in BSC-1 cells (African green monkey kidney cells) or FRhK-4 cells (Foetal rhesus kidney cells), HAV was demonstrated by

electron microscopy to be located in vesicular structures (Asher *et al.*, 1988). When liver biopsies from infected marmosets were examined by electron microscopy, similar structures were observed both in hepatocytes and Kupffer cells (Shimitzu *et al.*, 1978). The virus containing vesicles also appeared to be released from these cells into the blood and biliary system. The inability of wild type HAV to cause cytopathic effect *in vitro* also tends to suggest that this virus is unlikely to be directly cytotoxic *in vivo*.

Alternatively, the pathogenesis of the liver seen upon infection with HAV may be due to the immune responses of the host towards the pathogen. Pathological damage of the liver temporally coincides with the appearance of anti-HAV IgM in the serum. However, Slusarczyk *et al* (1985) showed that anti-HAV antibodies with or without complement are not responsible for hepatocyte destruction and Gabriel *et al* (1986) failed to detect cytotoxic antibodies in HAV infection. The cell mediated immune response was implicated in the destruction of infected cells by *in vitro* studies (Kurane *et al.*, 1985; Vallbracht *et al.*, 1984; Baba *et al.*, 1993). In these studies HAV infected cells were lysed by natural killer cells and cytolytic T cells.

The role of inflammatory cytokines and chemokines in the pathogenesis of HAV infection has not yet been established. Infiltration of the liver by T-lymphocytes and neutrophils upon infection with HAV suggests a role of chemotactic cytokines. Studies of chemokine response in respiratory syncytial virus (RSV) infection of respiratory epithelial cells showed the a potent neutrophil and T-lymphocyte attractant interleukin 8 (IL8), was produced by these cells (Noah and Becker, 1993). This IL8 secretion appeared to be stimulated by tumour necrosis factor (TNF) and IL1 excreted from RSV infected epithelial cells (Arnold *et al.*, 1994). Similar findings resulted from the study of cytokine release in rhinovirus infection (Subauste *et al.*, 1995). HIV infected alveolar

macrophages secrete the chemokine macrophage inflammatory protein 1 α (MIP-1α) resulting in an influx of cytotoxic T cells (Denis and Ghadirian, 1994). Infection of hepatocytes and Kuppfer cells may result in the secretion of such chemokines and thereby cause the infiltration of immune cells seen.

1.3 CLINICAL SYMPTOMS

The result of infection with HAV is extremely variable. In under 2 year olds, the course of infection usually remains asymptomatic. A study of children at day care centres suspected of transmitting the virus to older siblings, indicated that only 4-16% of pre-school children presented with symptoms upon infection with HAV. The majority of adults develop overt disease.

Symptoms of HAV infection are similar to those found with the other types of viral hepatitis and the course of illness may be divided into four clinical phases: (i) the incubation period before disease symptoms are apparent, (ii) the preicteric or prodromal phase, (iii) the icteric phase and (iv) the convalescent period. At the prodromal stage the first symptoms of infections with HAV are seen. The occurrence and severity of disease symptoms vary considerably between patients. The most common manifestations are fatigue (52-91 %), anorexia (42-90 %), abdominal pain (37-65 %), fever (32-73 %), nausea, vomiting (26-87 %), headache (26-73 %), myalgia (15-32 %), arthralgia (11-40 %), and rash (1-14 %) (Friedman and Dienstag, 1984). The occurrence of diarrhoea in 20% of infected adults and 60% of children implies that HAV replicates in the gut (Lemon, 1985). However the diarrhoea has been shown in some cases to be caused by another enteric infection acquired from the same source (Dismukes *et al.*, 1969). The onset of these symptoms occurs very quickly, often taking only 24 hours for an infected

individual to regress from apparent healthiness to acute illness. Serum levels of hepatic enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) become elevated several days after the onset of symptoms.

The icteric phase usually begins within 10 days of the commencement of disease symptoms. As levels of serum bilirubin increase to 5 - 15 mg / 100 ml serum, the urine becomes darkened indicating bilirubinuria followed by pale stools and the yellow jaundiced appearance of skin, mucous membranes, sclerae and conjunctivae. The liver may enlarge and become sensitive to palpation. In extreme cases (<1.5% of hospitalised icteric patients) the extent of necrosis may be so extensive that the function of the liver becomes severely impaired. Symptoms of this 'fulminant hepatitis' are sudden high fever, severe abdominal pain and vomiting. Patients may suffer seizures and become comatosed due to hepatic encephalopathy which leads to the death of 70-90% of those who develop the fulminant disease.

1.4 EPIDEMIOLOGY

The prevalence of hepatitis A correlates with hygiene and sanitary conditions. Table 1.1 shows the world wide prevalence of anti-HAV antibodies in the adult population. A decline in the prevalence of HAV in northern and western Europe and North America correlates with the development and related improvement in the sanitary conditions in these areas over the past several decades (Hadler, 1991). A similar pattern has been noted in other areas such as Australia, Japan and Hong Kong (Purcell, 1994). In developing countries where HAV is endemic, the virus is usually contracted during childhood and symptoms are usually mild whereas in the more industrialised areas that have a higher level of sanitation, infection usually occurs in adults. A modern risk of HAV infection is travel from low-prevalence areas to high-prevalence countries. A recent study showed geographical variation of age specific antibody prevalence to HAV and current incidence

of HAV infection in England (Gay *et al.*, 1994). Incidence of HAV infection as estimated by both seroprevalence and notification rates appears to be higher in Yorkshire and the North West than in the South East Thames region. This difference is thought to result from geographical variation in socio-economic status. The prevalence of anti-HAV antibodies in UK adult populations varies from 29 % in 20-24 year olds to 90 % in those over 60 years.

Table 1.1. Prevalence of anti-HAV antibodies in adult populations (Melnick, 1995)

Country of origin	% positive
Sweden	13
USA	43
Japan	50
Some South Pacific islands	52
Netherlands	52
Germany	55
Poland	60
Australia	62
Senegal	75
Greece	82
Taiwan	88
Kenya	88
Israel	94
South Pacific islands	95
Yugoslavia	97

The use of conventional immunological methods in the study of HAV with regard to the spread of individual epidemics is limited as this species of virus consists of only 1 serotype. The advent of gene amplification, molecular cloning and sequencing technologies has recently made comparison of different strains at the genome level possible (Jansen *et al.*, 1990). Sequence analysis revealed a variable region comprising of 168 bases at the putative junction of regions VP1 and 2A of the HAV genome (nucleotides 3024-3191) . When 171 individual isolates of HAV were examined, 4 distinct 'genotypes' (I-III and VII) of human HAV and 3 genotypes of simian derived HAV (IV-VI) emerged which differed from each other at >15% of base positions in this region (Table 1.2, Robertson *et al.*, 1991). Genotypes I and III have also been divided

into subgenotypes A and B. There do not appear to be any differences in the biological activities of the different genotypes of human HAV, however, the simian genotypes, isolated in Old World monkeys do not appear to cause disease in chimpanzees which are susceptible to human genotypes, whereas the human isolates do not routinely establish infection in Old World monkeys. The biological relevance of the different genotypes is therefore unclear.

Table 1.2. Geographic localisation of human HAV genotypes.

Area	Number of isolates	IA	IB	II	IIIA	IIIB	VII
North America	16	15	-	-	1	-	-
Central / South Americas	8	7	1	-	-	-	-
Europe	22	7	5	1	8	1	-
U.S.S.R	3	3	-	-	-	-	-
China	6	6	-	-	-	-	-
Southeast Asia, India, Japan	41	32	-	-	4	5	-
Australia, Africa, Middle East	8	1	6	-	-	-	1

1.5 VACCINATION

From 1944 (Stokes and Neef, 1945) until recently, prevention from HAV infection has been achieved by passive immunisation with a heterologous preparation of human immune serum globulin (ISG) which was shown to contain significant amounts of antibodies to HAV. Passive immunisation with ISG is generally recommended for travellers to endemic countries and close contacts in HAV outbreaks (Deinhardt, 1992). The major disadvantage of ISG immunisation is the duration of protection. A large dose of ISG (0.05-0.06 ml / kg) provides protection for 4 - 6 months (Conrad and Lemon, 1987; Weilland *et al.*, 1981). The concentration of HAV in ISG preparations varies according to the incidence of HAV among donors (Stapleton, 1992). The titre of anti-HAV found in commercial preparations ranges between 1:500 and 1:4000 by RIA (80-600 IU ml^{-1}). A reduction of the incidence of HAV infection in developed countries

resulting in a reduction in the titre of anti-HAV in ISG has recently caused concern that the effectiveness of this prophylactic treatment may decline (Stapleton, 1992). The first inactivated hepatitis A vaccine (Havrix, Smithkline Beecham Biologicals, Belgium) was introduced in Switzerland in 1991 and was subsequently licensed in several European countries (Andre *et al.*, 1992). The vaccine is derived from the HM175 strain of HAV adapted to growth in human diploid cells (MRC-5) and is inactivated by formaldehyde treatment. The recommended dose of this vaccine is well tolerated and produces almost 100 % seroconversion in healthy adults. To ensure long-term protection, a booster dose is administered 6 months to 1 year after the initial immunisation.

1.6 CLASSIFICATION

Biochemical and biological study of hepatitis A virus indicated that this virus should be classified as a picornavirus. The family picornaviridae consists of five genera, aphthoviruses, cardioviruses, enteroviruses, hepatoviruses and rhinoviruses. Hepatitis A virus has recently been designated the prototype of the new hepatovirus genera which also includes simian hepatitis virus (Minor, 1991). Previously, HAV was classified as an enterovirus since the primary route of transmission of this genera is the oral-faecal route. The new genera 'hepatoviruses' was suggested (Siegl and Lemon, 1990) due to differences in the thermal stability, nucleotide sequence, response to antiviral compounds, and behaviour in tissue culture of HAV when compared to other members of the family picornaviridae (Table 1.3) .

1.7 BIOPHYSICAL PROPERTIES

The hepatitis A virion is a non-enveloped, icosahedral particle with a diameter of 28nm (Feinstone *et al.*, 1973), a density of between 1.32 and 134 g / ml in caesium chloride and a sedimentation coefficient of 156-160 S in neutral sucrose solutions. The capsid is

composed of multiple copies of 4 different proteins. and contains a 7.48 kb single stranded RNA genome.

Table 1.3. Comparison of characteristics of the 5 genera of picornaviruses (Adapted from Hollinger and Ticehurst, 1990).

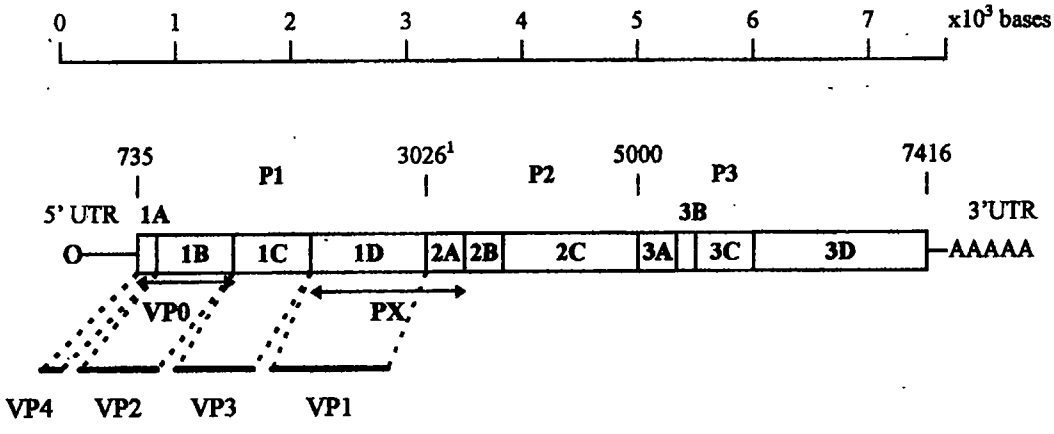
	Hepatovirus	Enterovirus	Aphthovirus	Cardiovirus	Rhinovirus
Prototype virus	HAV (HM175/wt)	Poliovirus type 1	Foot and mouth disease virus A12	Encephalo-myocarditis virus	Rhinovirus 14
Serotypes	1 (≥13 strains)	>70	7 (≥53 subtypes)	2 (≥ 6 strains)	>130
Primary hosts	Humans, other primates	Humans, other mammals	Cloven footed, other mammals	Mice, other mammals	Humans, other mammals
Primary habitat	Liver	Gut	Generalised	CNS, heart	Upper respiratory tract
Sensitivity					
Acid (pH3)	Stable	Stable	Labile	Stable	Labile
Heat (60 °C, 60 min)	Stable	Labile	Labile	Labile	Labile
Guanidine	Resistant	Sensitive	Resistant	Resistant	Sensitive
Disoxaril	Resistant	Sensitive	Resistant	Resistant	Sensitive
Biophysical properties					
Buoyant density (g/cm^3 in CsCl)	1.32-1.34	1.34	1.43-1.45	1.34	1.39-1.42
Sedimentation coefficient (S)	156-160	156-160	142-146	156	149
Genome					
Length (Kb)	7.48	7.44	8.4	7.84	7.21
% G + C	38	47	43	50	40
Poly C tract	-	-	+	+	-
Poly C+U	+	-	-	-	-
Structural Proteins (Mr $x10^{-3}$)					
VP1	33.2	33.5	23.3	31.7	32.4
VP2	24.8	30.0	24.7	29.0	28.5
VP3	27.8	26.4	24.3	25.1	26.2
VP4	≥2.5	7.4	8.5	7.2	7.2
VPg	2.4	2.3	2.6-2.7	2.2	2.4
Protein cleavage[1]					
Proteases	3C only	2A + 3C	2A + 3C + L	2A + 3C	2A + 3C
Primary cleavage site	2A/2B junction	VP1/2A junction	2A/2B junction	2A/2B junction	VP1/2A junction
Enzyme	3C	2A	2A	2A	2A

[1]Ref: Jia *et al.*, 1993; Schultheiss *et al.*, 1994; Martin *et al.*, 1995.

1.7.1 Genome

Provost *et al.* (1975) proposed HAV virions contained an RNA genome due to the cytoplasmic location of the virus and the effects of ribonuclease combined with heat treatment on infectivity. Electron microscopy (Siegl and Frösner, 1978a; 1978b) and centrifugation of radio-iodinated HAV nucleic acid molecules in sucrose gradients (Coulepis *et al.*, 1981) indicated that the genome is linear with a molecular weight of 2.25 x 10^6. The ability to infect cultured cells with naked HAV RNA (Locarnini *et al.*, 1981; Gauss-Müller *et al.*, 1984) suggested that the genome is positive sense. More detailed analysis was made possible by the cloning of the HAV genome by Ticehurst and co-workers (1983). Subsequent sequence analysis revealed the typical genome organisation of the picornavirus family (Najaran *et al.*, 1985; Cohen *et al.*, 1987), comprising of 3 regions: (i) an untranslated region (5' UTR) of 735 nucleotides at the 5' terminus, (ii) a large, single open reading frame of 6681 nucleotides encoding a polypeptide of 2227 amino acids and (iii) a 63 nucleotide 3' terminal untranslated region (3' UTR) which includes a poly-adenosine tail (fig 1.1).

Figure 1.1 Genome structure of HAV (Adapted from Weitz and Siegl, 1993)



[1] Dotzauer et al., 1995

The 5' UTR is essential for HAV replication and translation. Analysis of the 5' UTR revealed a highly ordered secondary structure which allows binding of ribosomes at an internal ribosomal entry site (IRES) between nucleotides 161-734, adjacent to a translation initiation codon (AUG) and directs cap-independent initiation of viral translation (Brown *et al.*, 1991; 1994; Glass and Summers, 1992). The majority of eukaryotic messenger RNA is translated after recognition of a 5' terminal cap structure by a translation initiation factor (eIF-4F) which allows binding of the 40s ribosomal subunit (Hershey, 1991). In enteroviruses and rhinoviruses, the 2A protein cleaves eIF-4F, thus terminating host cell protein synthesis whilst allowing viral translation to continue without competition for translational requirements (Kräusslich *et al.*, 1987; Lamphear *et al.*, 1993).

The 5' UTR contains two polypyrimidine tracts (C + U). The first is found between nucleotides 100-139 and the second near the initiation codon, nucleotides 706 to 726. Both of these polypyrimidine tracts appear to be involved in the initiation of translation (Silveira Carneiro *et al.*, 1995).

The region(s) within the 5' UTR responsible for RNA replication have not yet been defined (Jia *et al.*, 1996). Recently Kusov *et al.* (1996) , after analysis of interactions of the 3'UTR with host cell proteins, suggested that a putative pseudoknot structure in this region together with the 3D region encoding the viral RNA polymerase may be essential for the formation of a replication complex which initiates minus strand synthesis.

The coding region of the HAV genome can be divided into 3 regions (Figure 1.1). The four structural protein encoding sequences are found in region P1 adjacent to the 5' UTR. Region P2 encodes 3 non-structural proteins , 2A, 2B and 2C and P3 encodes non-structural proteins 3A, 3C and 3D and a further structural protein, 3B (VPg).

1.7.2 Structural proteins

The HAV capsid consists of multiples of 4 different proteins, VP1 (33 kDa), VP2 (25 kDa), VP3 (28 kDa), and VP4 (2.5 kDa). The presence of VP4 is assumed from comparison of the nucleotide sequence with that of other picornaviruses, however this protein has not yet been detected in HAV virions (Tesar *et al.*, 1993). The tertiary structure of the capsid proteins and their alignment in the capsid is unclear. The thermal stability of HAV suggests that the structure of the particle differs from that of the other members of the picornavirus family (Table 1.3). Analysis of the thermal stability of empty HAV capsids revealed them to have similar characteristics to those of poliovirus which infers that in HAV, the presence of RNA improves HAV particle stability (Ruchti *et al.*, 1991). A fifth structural protein is encoded in the P3 region of the genome. VPg (3B) is a small protein (2.4 KD) found to be covalently attached to HAV RNA in the capsid which may function in the initiation of negative strand RNA synthesis.

1.7.3 Non-structural proteins

Again, the non-structural proteins have been classified on the basis of comparison of the genome sequence with that of other picornaviruses. The sizes and putative functions of these proteins are shown in Table 1.4

Table 1.4. HAV non-structural proteins

Protein	Size (KD)	Function
2A	10.0	Unknown
2B	12.0	Transcription?
2C	38.4	Transcription?
3A	8.0	Unknown
3C	24.1	Protease
3D	56.3	Polymerase

1.8 VIRAL REPLICATION

1.8.1 Cell attachment, entry and uncoating

The first stage in viral infection is attachment of the virus to a specific cellular receptor located in the plasma membrane of the host cell. Once bound, the cell receptor mediates the internalisation and uncoating of the virus. The ability of a particular virus to infect a cell is partly determined by the expression of the corresponding receptor. HAV has been demonstrated to associate with several host cell macromolecules (Seelig *et al.*, 1984; Lemon and Binn, 1985; Margolis and Nainan, 1990; Zajac *et al.*, 1991), however it was not until recently that the probable functional receptor for HAV was finally identified in African Green Monkey Kidney cells and named HAVcr-1 (Kaplan *et al.*, 1996). Characterisation of the molecule showed HAVcr-1 to be a novel major histocompatibility complex (MHC) class I integral membrane molecule with an extracellular globular first domain and an extended mucin-like structure. On the basis of known binding of other picornaviruses to their host cell receptors, the authors suggested that HAV may bind to the first domain. The normal function of this receptor is unknown but may be involved in cell-cell signalling. A similar mucin-like receptor which appears to induce HAV attachment has also been identified on S.la/Ve-1 cells, a hybrid between marmoset liver cells and Vero cells (Ashida and Hamada, 1997). The *in vivo* and *in vitro* distribution of these putative receptors has yet to be determined. Several studies have shown that in order to infect cells in culture, HAV requires calcium ions which may interact with either the host cell receptor or the receptor binding domain of HAV, facilitating receptor-virus binding.

The process by which the receptor bound virus undergoes internalisation and uncoating is unclear. Early studies showed that interaction between poliovirus and it's cellular receptor altered the structure of the viral capsid abolishing infectivity (Joklik and

Darnell, 1961; Fenwick and Cooper, 1962). The 'A particle' thus formed is now considered to be a destabilised intermediate in cell entry (Fricks and Hogle, 1990). Formation of this particle is achieved by the loss of VP4 and externalisation of the N terminus VP1. Little is known about the events which occur during internalisation of HAV and the presence of VP4 in the capsid has not yet been conclusively established. Further study of HAV-receptor interactions are required to elucidate the mechanism by which the naked viral genome appears in the cytoplasm of infected cells.

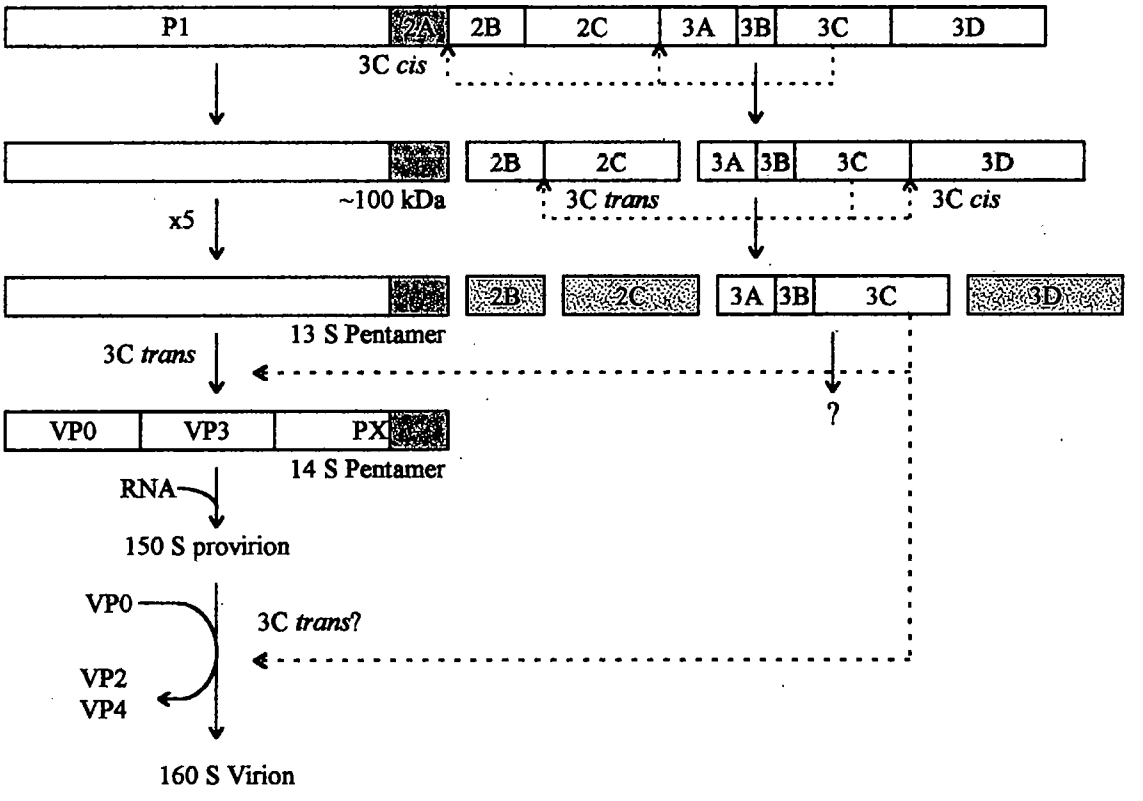
1.8.2 RNA synthesis

The initial step in the replication of the genome of picornaviruses is transcription of the positive strand RNA to form negative strand RNA. From this template, multiple copies of the genome can be synthesised. Studies of poliovirus replication (Lama *et al.*, 1994; Giachetti *et al.*, 1992) have shown that proteins involved in the synthesis of negative strand picornaviral RNA include viral proteins $3D^{pol}$, 3CD, VPg (3B) and its precursor 3AB, as well as at least one host cell protein factor (p36). The replication complexes comprise proteins interacting with a 3' terminal RNA cloverleaf structure of poliovirus and are associated with smooth membrane in the cytoplasm (Caliguiri and Tamm, 1970). VPg or a polyurydylated form of its precursor 3AB may act as a primer in the initiation of polymerisation by $3D^{pol}$ (Takeda, *et al.*, 1986; Takegami *et al.*, 1983) . Protein 3AB has also been demonstrated to activate $3D^{pol}$ (Lama, *et al.*, 1995). Negative strand RNA is readily detectable in poliovirus infected cells whereas this replicative intermediate has not been convincingly demonstrated upon HAV infection of cultured cells, hence little information is available regarding the mechanisms involved in HAV RNA replication.

1.8.3 Translation and polyprotein processing

As with all picornaviruses, the HAV genome is translated in a single open reading frame to potentially form a single, large polypeptide. However, cleavage of the nascent polypeptide precludes the formation of the complete molecule. All cleavages of the HAV polypeptide are thought to be catalysed by the viral encoded protease 3C. Unlike enteroviruses and rhinoviruses, the primary cleavage of the HAV polyprotein is thought to occur at the 2A/2B junction releasing a molecule of 98.5 kDa composed of the 4 structural proteins and non-structural protein 2A (Jia *et al.*, 1993; Schultheiss *et al.*, 1994; Martin *et al.*, 1995). Borovec and Anderson (1993) proposed a model for the synthesis and assembly of HAV structural proteins based on the results of pulse-chase experiments (Figure 1.2). After the initial cleavage of the HAV polyprotein at the 2A:2B junction, the P12A polyprotein rapidly form a 13 S pentamer. Each polypeptide in the pentamer is then cleaved by 3C in *trans* at the VP2:VP3 and VP3:VP1 junctions to form a 14 S pentamer containing VP0 (VP4 and VP2), VP3 and PX (VP1 and 2A). RNA is then incorporated and the polyprotein PX is cleaved to release protein 2A and a 150 S provirion is formed. A mature 160 S virion is formed when VP0 is cleaved by protein 3C to give VP4 and VP2. Study of the synthesis of non-structural proteins revealed the cleavage of proteins 2B, 2C and 3D, but the processing of the remaining 3ABC polyprotein has yet to be elucidated (Jurgensen *et al.*, 1993; Schultheiss *et al.*, 1995). The cleavage of 3D from the P3 polyprotein tends to suggest that unlike that of poliovirus, the precursor 3CD is not formed and therefore is not involved in the synthesis of HAV negative strand RNA or cleavage of the structural proteins. However, these conclusions are drawn from *in vitro* translation studies and therefore may not strictly represent the events that occur *in vivo*.

Figure 1.2. Post translational processing of HAV proteins (Adapted from Borovec and Anderson, 1993; Jurgensen et al., 1993; Schultheiss et al., 1995)



1.9 REPLICATION IN TISSUE CULTURE

HAV differs most strikingly from the other members of the picornavirus family in its ability to replicate in tissue culture. Picornaviruses typically replicate rapidly in tissue culture showing cytopathic effects and can produce up to 10^5 infectious particles per cell within 5-10 hr of infection (Baltimore *et al.*, 1966). Despite the long history of HAV, successful propagation of the virus in tissue culture was not achieved until 1979 when Provost and Hilleman (1979) demonstrated the replication of HAV in primary liver cell culture and foetal rhesus kidney cells. No cytopathology was seen in the infected cells and immunofluorescent detection was necessary to demonstrate replication of the virus. HAV has subsequently been propagated in a small variety of cells including those derived from African green monkey kidney (BSC-1, Vero and BGMK), foetal rhesus kidney (FRhK4, FRhK6 and Frp/3) and human hepatoma (PLC/PRF/5), human diploid

lung (MRC5) as well as primary African green monkey kidney cells and primary human fibroblasts (Frösner *et al.*, 1979; Flehmig, 1980; Daemer *et al.*, 1981; Flehmig *et al.*, 1981; Gauss-Müller *et al.*, 1981; Locarnini *et al.*, 1981; Siegl *et al.*, 1984; Vallbracht *et al.*, 1984; Binn *et al.*, 1984). Isolation of HAV from clinical specimens may take several months and serial passage is required before the virus becomes progressively more adapted to growth *in vitro* (Wheeler *et al.*, 1986). The majority of these tissue culture adapted variants establish persistent infection of cells following 4-8 days of logarithmic growth and show very little effect on host cell morphology or protein synthesis (De Chastoney and Siegl, 1987). Electron microscopy revealed accumulation of multilayered membranes and virus containing vesicles in the cytoplasm of infected cells (Kiernan *et al.*, 1987, Asher *et al.*, 1987; 1988). The majority of virus remains cell associated.

Table 1.5. Cell culture adapted, cytopathic strains of HAV

Strain	Origin	Passages in cell culture	Cell line	Plaque purification	Reference
HM175/18f	HM175/wt[1]	16 passages + 1 year persistent infection	BSC-1	-	Lemon *et al.*, 1991
HM175A.2	HM175/wt	35	BSC-1	3 rounds	Anderson *et al.*, 1987 Anderson, 1987
pHM175	HM175/wt[1]	10 ~38	AGMK[2] primary cells BSC-1 cells	2 rounds	Cromeans *et al.*, 1987; 1989
FG	FG (Italy)	3	Frp/3	-	Morace *et al.*, 1993
S10	S10 (Italy)	3	Frp/3	-	Morace *et al.*, 1993

[1]HM175 wild type + 6 marmoset passages
[2]African green monkey kidney cells

Cytopathic variants of HAV have been isolated by repeated subculture of persistently infected cells, acute passage of virus and plaque purification (Table 1.5). These

cytopathic variants replicate a little faster than other cell culture adapted strains but still produce low yields of virus. Infection of cells with cytopathic HAV variants does not cause shut down of host cell protein synthesis as observed with many other cytopathic picornaviruses. The cytopathic effects seen include pyknosis, aggregation of cells and detachment from the surface of the flask and includes the majority of infected cells.

1.9.1 Adaptation of HAV to growth in cell culture

The genome changes required for improved growth of HAV in tissue culture have been studied for several cell culture adapted variants. Mutations in the P2 (proteins 2B and 2C), P3 and 5' untranslated regions of the of HM175/wt resulted in a cytopathic, rapidly replicating variant, HM175/18f (Zhang *et al.*, 1995). Mutations in only the P3 and 5' UTR regions of HM175 did not cause cytopathology but when placed on a background of the P2 mutations, larger foci of cytopathology were observed. Zhang and co-workers proposed that interactions between proteins 2B and 2C and the 5' UTR of HAV may occur in the formation of a membrane-bound HAV replication complex. Again, changes in proteins 2B and 2C as well as in the 5' UTR and P3 region (particularly protein 3A) resulted in the adaptation of several strains of HAV to growth in cultured monkey kidney cells and MRC-5 cells (Graff *et al.*, 1994a and 1994b; Emerson *et al.*,1992; Morace, *et al.*, 1993; Day *et al.*, 1992; Funkhouser *et al.*, 1994). Several of the mutations seen are present in a variety of cell culture adapted strains regardless of origin or cell culture system (Table 1.6).

Table 1.6. Common nucleotide changes occurring during adaptation of HAV to growth in tissue culture.

Genome region	Nucleotide position	wild type[1]	HM175 p16[2]	HM175 18f[2]	HAV/7[2]	GBM/ FRhK4[3]	FG[4]
P2 (2B)	3889	Ala	Val	Val	Val	Val	Val
P2 (2C)	4426	Lys	Thr	Lys	Lys	Thr	Thr
P3 (3A)	5010-5012	Asp	Asp	del	Asp	Asp	del
P3 (3A)	5013-5015	Asp	Asp	Asp	Asp	del	del
P3 (3A)	5016-5018	Asp	Asp	Asp	Asp	del	del
P3 (VPg)	5232	His	His	His	Tyr	Tyr	His

[1] Based on HM175 and GBM wild type nucleotide sequences
[2]Ref: Zhang *et al.*, 1994
[3]Ref: Graff *et al.*, 1994a
[4]Ref: Morace *et al.*, 1993, Beneduce *et al.*, 1995

Despite many studies, the mechanisms hampering the replication of HAV in tissue culture have yet to be clarified. It has been suggested that asynchronous uncoating and replication of HAV causes the appearance of slow replication in *vitro* (Wheeler *et al.*, 1986; Cromeans *et al.*; 1989 Cho and Ehrenfeld, 1991). Alternatively, Anderson *et al.* (1988) showed highly efficient packaging of HAV RNA into virions and proposed that the number replicative intermediates available for further replication was therefore diminished at an early stage in the growth cycle. Further study implied that due to low levels of viral RNA replication early in the growth cycle of HAV, structural proteins form empty capsids. However, later in infection, levels of 3C protease increase concurrently with viral RNA. High levels of the protease lead to cleavage of capsid proteins prior to formation of 13 S pentamers instead producing 5 S monomers which cannot self assemble into virions and are degraded (Borovec and Anderson, 1993).

1.10 ANIMAL MODELS

Since the 1950's, there have been many attempts to transmit HAV to non-human primates such as chimpanzees, macaques, sooty mangabeys, African green monkeys, baboons and gibbons (reviewed by Gust and Feinstone, 1988). The early studies were hampered by the techniques then available for analysis of infection, the acquisition of animals from the wild and subsequent unhygienic conditions and the limited understanding of the animals used. None of these studies convincingly showed reproducible transmission of the virus to the animals. A large investigation of viral hepatitis in 37 chimpanzees gave an indication that these primates were susceptible to HAV (Deinhardt *et al.*, 1962). Although the study was complicated by the possible presence of both HAV and HBV in the inocula making the results inconclusive, valuable information regarding the normal liver function of chimpanzees was obtained. During the early 1960's, colonies of white-lipped (*Saguinus nigricollis*) and cotton topped (*S. oedipus*) marmosets were established by Deinhardt and Deinhardt (1966). The use of these colonies allowed selection of experimental animals with a known medical history. In 1967, Deinhardt and colleagues reported the transmission of the virus from serum or plasma of patients with hepatitis during the acute phase of illness to the white-lipped marmosets. Of the five sera inoculated, one, designated GB was found to be highly infectious to tamarins and could not be neutralised by post hepatitis A convalescent serum. Two distinct infectious agents were isolated from infected tamarins (GBV-A and GBV-B). Based on nucleotide and amino acid sequencing, these viruses have been classified as flaviviruses and may possibly be related to hepatitis G virus (Simons et al., 1995; Schlauder et al., 1995). In a cross challenge study using the MS-1 agent isolated during studies at the Willowbrook state school (Krugman *et al.*, 1967) and the GB agent, animals which were pre-inoculated with the MS-1 agent were susceptible to infection when inoculated with GB sera and vice versa. The ability to infect marmosets with the MS-1 agent was

subsequently confirmed (Lorenz *et al.*, 1970). Mascoli and co-workers (1973) successfully inoculated tamarins (marmosets, *S. mystax* and *S. nigricollis*) with blood from a Costa Rican patient inducing hepatitis as characterised by increased enzyme levels and liver histopathology within 25-55 days of inoculation. The detection of HAV strain MS-1 in stools of infected patients by immune electron microscopy (Feinstone *et al.*, 1973) provided Dienstag and colleagues (1975) with a more specific method of demonstrating infection of the chimpanzees (*Pan troglodytes*) with HAV. Transmission of HAV to other non-human primates including lesser bushbabies (*Galago senegalensis*, Grabow and Prozesky, 1975), red bellied tamarins (*S. labiatus*, Purcell and Dienstag, 1978; Peterson *et al.*, 1979), stump-tailed monkeys (*Macaca speciosa*, Mao *et al.*, 1981), owl monkeys (*Aotus trivirgatus*, Lemon *et al.*, 1982; LeDuc *et al.*, 1983) and cotton eared marmosets (*Callithrix jacchus*, Shibayama, 1985) has now been achieved.

The most extensively studied animal models for HAV infection are tamarins and chimpanzees as infection is highly reproducible and follows a similar course to that of man. Infection of lesser bushbabies with HAV resulted in a similar response to that seen in man, chimpanzees and tamarins, however, this response was not seen in all infected animals suggesting that these primates are less susceptible to HAV (Grabow *et al.*, 1981). Both stump tailed and owl monkeys are susceptible to infection with HAV. Again, experimental infection in these animals results in a milder form of the disease than seen in man.

1.10.1 Experimental hepatitis A in tamarins and chimpanzees

Tamarins (*Saguinus spp.*) may be productively infected with HAV via parenteral or oral inoculation, the most susceptible species being *S. mystax, S. labiatus (rufiventer)* and *S. fuscicollis.* The resulting disease is generally milder than that seen in man and is comparable to that of chimpanzees (*Pan troglodytes*). Clinical features of HAV infection

of non-human primates include anorexia, lassitude, ruffled hair and diarrhoea, but these are unreliable markers of disease (Dienstag *et al.*, 1975; Margolis *et al.*, 1988). Biochemical and histopathological analysis is required in studies involving these animals. The incubation period usually ranges from 17-28 days. HAV antigen or infectious particles may be detected in the faeces several days before the serum enzyme levels begin to rise (Krawczynski *et al.*, 1981; Maynard *et al.*, 1975; Schulman *et al.*, 1976). The onset of faecal shedding is usually sudden and reaches a maximum level when histological changes associated with acute hepatitis become apparent and then begins to decline rapidly (Dienstag *et al.*, 1975; Ticehurst *et al.*, 1987). Viraemia generally occur at the same time as faecal shedding but the titres of HAV detected in serum are several fold lower than those in the faeces (Lemon *et al.*, 1988; Cohen *et al.*, 1989). The dose of virus administered does not appear to affect the severity of disease, but a linear correlation between the titre of the inoculum and the incubation period has been demonstrated (Gust and Feinstone, 1988).

Biochemical analysis reveals elevations in liver enzyme activity in the serum of infected animals with peak activity coinciding with the detection of HAV antigen in the liver. The enzymes generally assayed are isocitrate dehydrogenase (ICD), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). However, since elevations of ICD and AST are noted in other illnesses, assay of serum ALT is regarded as being more informative. In two studies, Karayiannis and co-workers (1986; 1990) found the baseline serum ALT to range between 8 and 18 IU / l in tamarins whereas in a study of chimpanzees the mean pre-incubation level was found to be 38 IU / ml (Karron *et al.*, 1988). Serum ALT levels rise to a maximum of between 120-275 IU / l, however occasionally much higher levels are detected (Karron *et al.*, 1988; Margolis *et al.*, 1988; Karayiannis *et al.*, 1986; 1990; Emerson *et al.*, 1992). There does not appear to be any difference between chimpanzees and tamarins in peak ALT levels, but interpretation of

the available data is difficult as slight alteration of assay temperature has a profound effect on the results obtained. Therefore, comparison of data from different studies may not be satisfactory.

Histological analysis of liver from HAV infected animals show similar changes to that of human patients. Whilst the livers of these primates are generally damaged to a lesser degree compared with the changes observed in human liver, occasionally severe necrosis is noted. The most prominent feature in diseased livers is mononucleocytic, periportal infiltration. Hepatocyte degeneration is milder than that seen in humans and is often limited to the periphery of lobules. As the disease progresses, cells in the bile ductules proliferate and mononuclear cells infiltrate the duct walls. An iron pigment may accumulate in sinusoidal lining cells, particularly macrophages in the portal tracts. As recovery begins, the characteristic signs of regeneration seen are mitotic and multinucleated cells. Electron microscopy reveals aggregated HAV particles within vesicles associated with multiple myelin-like membranes in the cytoplasm and the formation of dense heterochromatic granules in the nuclei of hepatocytes. Shimizu and colleagues (1978) counted between 10 and 80 virus particles in infected hepatocytes and also noted particles in sinusoidal cells. Immunofluorescence also demonstrated the presence of HAV antigen in these cells (Mathieson *et al.*, 1980; Krawczynski *et al.*, 1981).

Karayiannis and co-workers (1986) studied the host immune response to HAV in tamarins (*S. labiatus*). Circulating anti-HAV IgM was detected within 3 weeks of inoculation, coinciding with the peak of serum ALT activity. The IgM response was maximal at 4-5 weeks post inoculation and then decreased rapidly. By the seventh week when anti-HAV IgM was no longer detectable an IgG response to the virus could be measured.

1.11 TRANSMISSION OF HEPATITIS A VIRUS VIA BLOOD PRODUCTS

Until recently, transmission of HAV via blood or blood products has been extremely rare (Barbara *et al.*, 1982; Hollinger *et al.*, 1983; Weisfuse *et al.*, 1990). The viraemic period for HAV is transient, only lasting up to 2 weeks and the risk of transmission by this route had been considered to be negligible. It was therefore surprising when in 1992 an outbreak of hepatitis A amongst patients with haemophilia was attributed to a factor VIII preparation used to treat their illness (Mannucci, 1992). A total of 52 patients visiting 12 haemophilia centres throughout Italy contracted the disease between 1989 and 1992. All but one of these patients suffered with severe haemophilia A and required regular high quantity infusion of clotting factor. They had each been infused, within 2 months of onset of symptoms, with a factor VIII concentrate (Emclot Octa VI) which had been manufactured in Italy by AIMA Derivati from plasma pools collected from paid plasma donors in the United States. Prior to fractionation, the plasma had been tested for hepatitis B antigen, anti-HIV and raised ALT levels. The concentrated clotting factor was also treated with a mixture of lipid solvent (tri-(n-butyl) -phosphate) and detergent (Polysorbate 80) in order to inactivate any contaminating viruses. Subsequent detailed study of 29 of these patients compared with 71 randomly chosen control patients with haemophilia indicated that HAV had been transmitted via factor VIII and the authors proposed that patients with haemophilia be vaccinated against HAV (Mannucci *et al.*, 1994). PCR analysis of 9 lots of the factor VIII thought to be responsible for transmission of HAV to 4 patients revealed that 4 lots contained HAV genomic sequences and that each of the patients had been transfused with contaminated material. The sequences of the VP3 region of HAV RNA amplified from the different lots of clotting factor were 96-99% homologous and sequences amplified from the serum of each of the 4 patients were identical to that in the contaminated lot of factor VIII that they received. This was confirmed by amplification and sequencing of the VP1/2A junction of HAV isolated

from one of the patients and from the contaminated factor VIII preparation received by that patient (Purcell *et al.*, 1994). Further reports of transmission of HAV to haemophiliacs throughout Europe soon emerged (reviewed by Vermylen and Peerlink, 1994).

Gerritzen and co-workers (1992) reported acute hepatitis A in patients with haemophilia in Bonn, Germany, between 1987 and 1992. Of 46 patients receiving a factor VIII preparation (Octavi, Octapharma, Dusseldorf) which had undergone the same virus inactivation procedure as that implicated in the Italian outbreak, 13 contracted acute HAV infection during this period. HAV RNA was amplified by nested PCR in 1 of the batches of factor VIII given to the patients (Normann *et al.*, 1992). This prompted a retrospective study of HAV seroconversion amongst patients with haemophilia and von Willebrand's disease at this centre (Brackmann *et al.*, 1994). The showed that of 29 susceptible patients who had only received the solvent/detergent treated clotting factor, 11 seroconverted, and of 27 patients receiving both solvent/detergent treated and ion exchange chromatography purified concentrates, 6 seroconverted. In contrast, of 139 susceptible patients receiving pasteurised clotting factors only 1 seroconverted and this was likely to have resulted from contact with an infected sibling.

A third outbreak of hepatitis A amongst haemophiliacs occurred at a centre in Leuven, Belgium, (Peerlink and Vermylen, 1993). All of the 250 patients with haemophilia A were transfused with solvent/detergent treated factor VIII manufactured by Biotransfusion (Lille, France) from 1991 until April, 1992 when the production site was changed to Octapharma (Vienna, Austria). Between May and November 1992, 5 of the patients presented with clinical hepatitis A and 1 further patient was found to have

seroconverted in subsequent screening. Each of these patients were being treated for severe haemophilia A by transfusion of very large amounts of factor VIII.

The final outbreak of hepatitis A amongst European haemophiliacs that has occurred to date was located in the Republic of Ireland (Temperley *et al.*, 1993; Lawlor *et al.*, 1994). During the months of May to December, 30 patients with haemophilia A became infected with HAV. Solvent detergent treated factor VIII, Octavi™, fractionated by Octapharma, Vienna from Irish plasma donations, was used exclusively from 1990 onwards. The 3 implicated batches were prepared from a single plasma pool. It was subsequently discovered that an outbreak of hepatitis A had occurred specifically in an area from which a portion of this plasma had been collected.

More recently, in the United States transmission of HAV via clotting factors has also been documented (MMWR, 1996). In the latter half of 1995, 3 recipients of Alphanate™ factor VIII concentrate (Alpha Therapeutic Corporation , California) and one recipient of Alphanine™ factor IX, produced by the same company, presented with hepatitis A. Amplification and nucleic acid sequencing of the VP1 region of the HAV genome revealed that 2 of the factor VIII recipients were infected with HAV genotype 1A which was also present in the implicated lot of Alphanate. The sequences found in each of the three PCR products were identical. Both of the clotting factor concentrates used by the patients were prepared using the solvent/detergent method of virus inactivation.

Concerns as to the safety of blood products with regard to HAV as well as other non-enveloped viruses have arisen due to these outbreaks amongst patients with haemophilia. The routinely used virus inactivation procedures, particularly solvent/detergent treatment need to be re-evaluated and where necessary, alternative or additional steps should be taken to ensure the safety of these medicines. Vaccination has been adopted in several

countries as an alternative approach to protect blood product recipients against HAV infection. However, this does not address the issue that not all viruses are inactivated by the solvent/detergent methods currently employed by certain manufacturers.

1.11.1 Steps taken to ensure safety of plasma products with regard to viruses

Several medicinal products are derived from human plasma including clotting factors (e.g. factor VIII, factor IX and fibrinogen), albumin, fibronectin and immunoglobulins. Since the 1940's, a viral inactivation step has been used in the preparation of such products. The risk of parenteral transmission of the naturally blood borne viruses, hepatitis B, C and Delta viruses and HIV, is of major concern. A viraemic phase occurs during infection with most viruses, therefore, there is also a small risk of parenteral transmission of those viruses such as HAV which are not usually considered to be blood borne. Transmission of HAV and parvovirus B19 by plasma products has been documented. In order to the reduce the risk of transfusion of virally contaminated products, manufacturers use plasma which has been screened for HCV, HBV and HIV antigen or antibodies and perform viral elimination or inactivation procedures during the preparation of plasma products. However, plasma is not presently screened for HAV or parvovirus B19.

1.11.1.1 Donor selection

The source of plasma from which the above derivatives are made is volunteer or remunerated human donors collected either as whole blood or more frequently from remunerated donors by plasmapheresis. It has been noted that the risk of transmission of viruses in products manufactured from plasma of non-remunerated donors is lower than that of products from paid donors (Leikola, 1993). However, it is not possible in some

countries such the United States, for the demand for plasma products to be met by unpaid donors alone.

When the transmission of HIV via blood became apparent, so did the need to prevent high risk individuals from donating blood. In the early 1980s, as well as raising the public awareness of AIDS, a donor selection process was set up. Prospective donors are now asked to complete a self assessed health check, directly questioned to determine whether the donor is in an 'high risk' group and examined for signs of intravenous drug abuse. This process helps to identify those at risk of transmitting hepatitis B, C and Delta viruses as well as HIV.

1.11.1.2 Plasma screening

Donated blood or plasma is routinely tested for HIV and hepatitis B and C infection. The test for hepatitis B introduced in the early 1980s was a radioimmunoassay of hepatitis B surface antigen which is present at high levels in the plasma of infected individuals. Subsequently testing of all donations for antibodies to HIV in 1985 and HCV in 1990 was introduced in the UK when commercial kits became available for routine testing for these viral markers. These assays are however limited by being unable to discriminate samples taken at the initial stages of infection. The time taken from infection with a virus until a marker of the infection such as antibodies, antigen or genome, can be detected is called the 'window period'. Using the currently available antibody marker tests, the window period for HIV has be estimated to be 22 -32 days however, the introduction of a test for HIV p24 antigen may reduce this time to 10-12 days (Dodd, 1996). In the case of hepatitis B, the assay currently used in screening of blood donations detects HBV surface antigen. The window period associated with detection of this antigen is 37-87 days (Mimms et al., 1993). Based on retrospective study of transfusion transmitted cases of

HCV, the window period for detection of antibodies to this virus has been estimated to be 54-192 days (Busche *et al.*, 1995; Lelie *et al.*,1992). While selection of donors and screening of blood and plasma reduces the risk of a plasma product being contaminated, the safety of the plasma cannot be guaranteed. The screened viruses, HBV, HCV and HIV, may escape detection during the window period and other viruses for which there is no routine testing, for example, Parvovirus B19 and HAV, may be present in the starting material. The introduction of virus elimination or inactivation during manufacture is an additional step to ensure the virological safety of blood products.

1.11.1.3 Virus elimination procedures

An approach to ensuring the virological safety of blood products is to introduce a procedure for elimination of any contaminating virus during manufacturing. The methods currently employed are affinity chromatography (Schreiber, 1989), anion exchange chromatography (Burnouf, 1991) and membrane filtration (Burnouf-Radosevich, 1994). In affinity chromatography purification, the plasma cryoprecipitate flows through an affinity column allowing the desired blood product to bind to specific antibodies and the column is then extensively washed before the purified protein is eluted in an appropriate buffer. Anion exchange chromatography is based on electrostatic interactions between the desired product and an exchange resin such as diethylaminoethyl cellulose (DEAE) or sepharose. At a specific pH and salt concentration, the desired protein will be held within the exchange resin whereas contaminating molecules or virus particles with a different isoelectric point flow through. After extensive washing with the appropriate buffer, the target protein may be eluted from the resin by altering the pH. Affinity chromatography appears to be more effective in virus elimination than ion exchange chromatography, however validation experiments show that the efficiency of either of these techniques vary depending on the virus used.

The efficacy of membrane filtration in virus elimination depends on the pore size of filter. Microporous hollow fibre filters capable of removing viruses as small as 25-30 nm such as HAV and parvovirus B19 are available, however, they may only be used with products smaller than 10 kDa e.g. factors II, IX, X and XI. Filters of larger pore size are required for factor VIII (100 kDa) and IgG (160 kDa).

1.11.1.4 Virus inactivation procedures

Contaminating viruses are routinely inactivated physically, by heating either dry or wet (pasteurisation), or chemically, by solvent detergent treatment. Dry heat treatment has the advantage that the lyophilised blood product may be heated severely (e.g. 80°C / 72 hours) and this inactivation step can be performed on the final product with no requirement for further processing. The efficiency of viral inactivation using terminal dry heat may however be reduced by increased stability of the lyophilised virus and incomplete drying of the samples. Solvent/detergent virus inactivation involves the addition of solvent (usually tri-(n-butyl) -phosphate) and detergent (usually Tween-20) during the manufacture of the blood product. The chemicals are subsequently removed by further processing in a virus free environment. This method of virus inactivation works by dissolving the lipid envelope of contaminating viruses, however, viruses such as HAV and parvovirus B19 are not affected as they do not posses an outer lipid envelope. In order to overcome this problem, the solvent/detergent method may be combined with heat treatment.

1.12 QUANTITATION OF HAV

The slow, non-cytopathic growth of the majority of strains of HAV has made detection and quantitation of the virus difficult. Prior to adaptation to growth in tissue culture, HAV antigen and antibodies against HAV were quantitated by radioimmunoassay (RIA, Hollinger *et al.*, 1975; Purcell *et al.*, 1976). Samples are incubated in wells of microtitre plates previously coated with convalescent serum from a patient with hepatitis A. Antibodies to HAV, radiolabelled with ^{125}I, are then added to the wells and the washed wells are cut out and placed in a gamma spectrometer for quantitation of bound radioactivity. Samples with gamma counts above a calculated cut-off are deemed positive. This assay is limited in the fact that it does not distinguish between non-infectious HAV antigen and infectious virus particles.

The development of a tissue culture based assay by Lemon and co-workers in 1983 significantly aided the study of HAV. This radioimmunofocus assay (RIFA) is a combination of the traditional cytopathic plaque assay and detection with radiolabelled antibodies. Cells grown in petri dishes are inoculated with the sample and incubated with an agarose overlay for up to 2 weeks. The overlay is then removed and the foci of infection detected by incubation with ^{125}I-labelled anti-HAV antibodies followed by autoradiography. The RIFA is useful in that it quantitates only cell culture infectious virus and has become a popular method in the study of HAV, however, the technique is extremely labour intensive and time consuming. Fluorescently labelled antibodies have subsequently been used to demonstrate foci of infection in a similar assay (Seigl *et al.*, 1984). More recently, several assays have been reported in which cells in microtitre plates are incubated with serial dilutions of HAV and viral antigen detected using labelled antibodies to give a measure of virus titre in 50 % infectious doses ($TCID_{50}$) / ml. These assays are also complex as they rely on either enzyme immunoassay (Yap and Lamb, 1994), fluorescent antibody detection and microscopic analysis (Nadala and Loh,

1990) or adsorbing, washing and eluting radio-iodinatied antibodies from individual wells prior to scintillation analysis (Krah, 1991). Each of these infectivity assays involve the maintenance of cells for long periods followed by careful manipulation in order to retain the integrity of cell monolayers during immunodetection.

In 1985, a strain of HAV was reported to produce cytopathic effects in cell culture (Venuti *et al.*, 1985). This fast growing (FG) strain was isolated from an outbreak of hepatitis A in Southern Italy. During the third passage in Frp-3 cells (derived from FRhK-4 cells, a foetal rhesus monkey kidney cell line), after 7-9 days post infection, cytopathic effects were observed. Subsequently, several cytopathic variants of have been obtained by repeated passage of HAV strain HM175 (HM175A.2, Anderson, 1987; pHM175, Cromeans *et al.*, 1987; 1989; Nasser and Metcalf, 1987).

These cytopathic strains of HAV made the development of cytopathic plaque assays possible and thereby greatly facilitated the study of this virus in vitro. The use of these cytopathic plaque assays is however limited to the study of the small number of cytopathic strains available. Since the adaptation of HAV to growth in tissue culture is associated with attenuation of the virus (Provost *et al.*, 1982; Karron *et al.*, 1988), information gained regarding the replication of these highly adapted strains may not be applicable to that of wild type strains *in vitro*.

An alternative approach to detection and quantitative assay of HAV is the specific amplification of the genome. The polymerase chain reaction, as originally developed by Mullis (Saiki *et al.*, 1985; Mullis *et al.*, 1986; Mullis and Faloona 1987), allowed amplification of specific sequences of DNA using a bacterial DNA polymerase (Klenow

enzyme) and oligonucleotide primers specific to the desired region of the genome to be amplified.

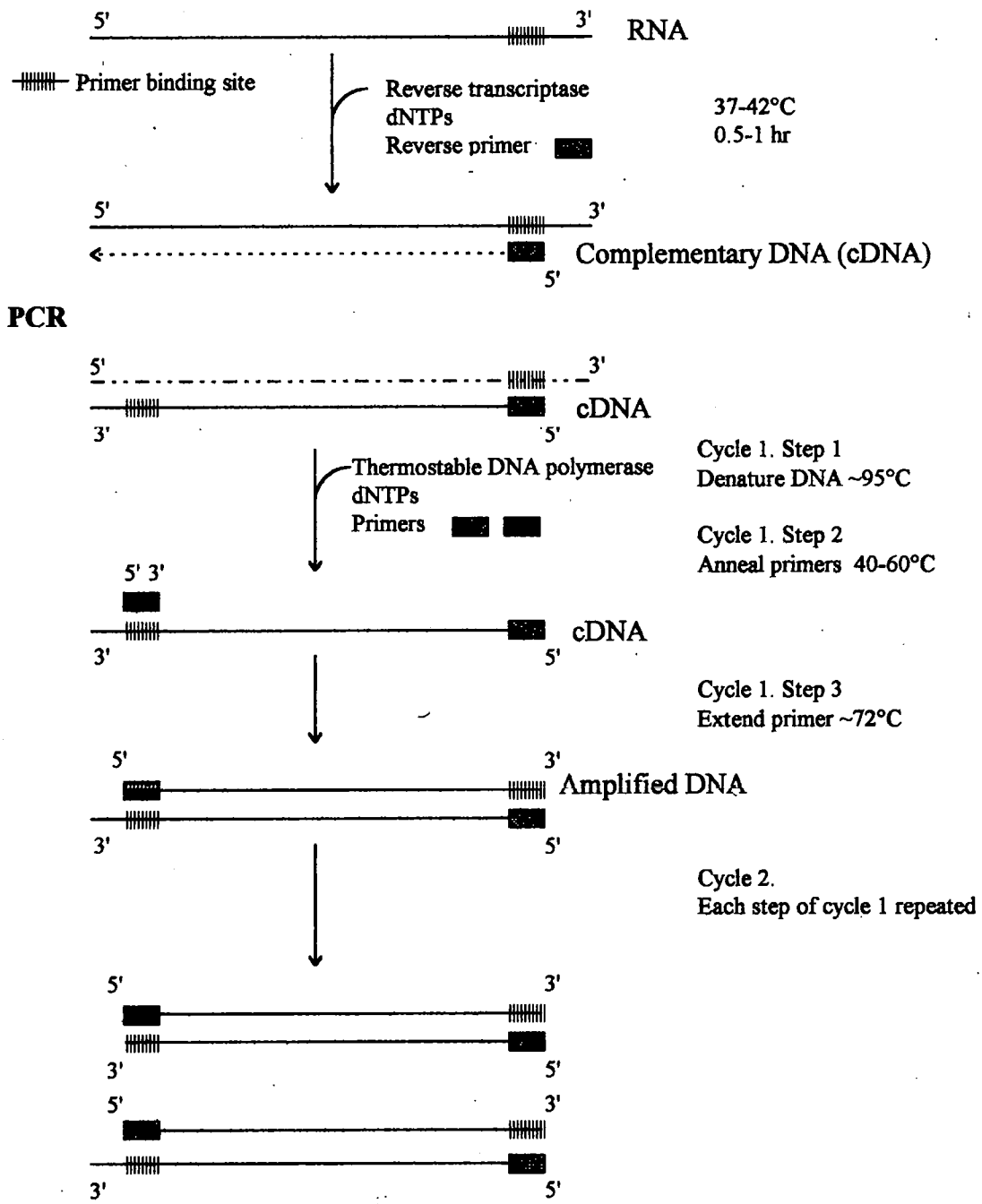
Extracted DNA was mixed with oligonucleotide primers in a buffer containing deoxyribonucleotides (dNTPs) and magnesium chloride at optimised concentrations. The mix was incubated at a high temperature, usually about 95 °C, for several minutes in order to denature the double stranded DNA. The temperature was then reduced to 37 °C before adding the Klenow enzyme to the reaction mix. At this temperature, the oligonucleotide primers could bind to the sample DNA allowing polymerisation of a complementary strand to occur. The samples were once again heat denatured and cooled to 37°C before adding a fresh aliquot of polymerase. This process was repeated until the amount of amplified DNA was large enough to be visualise by agarose gel electrophoresis.

Whilst PCR became a useful tool in molecular biology the method was extremely labour intensive and suffered with problems of specificity because the temperature of polymerisation was often lower than the optimal temperature for specific annealing of oligonucleotide primers. The technique was vastly improved by the introduction of thermostable DNA polymerase extracted from thermophilic bacteria such as *Thermus aquaticus* (Taq polymerase). This enzyme can withstand the heat denaturing temperatures involved in PCR and therefore need only be added to the reaction mix at the beginning thereby simplifying the technique and reducing the risk of contamination. The optimal temperature for Taq polymerases is between 70-75 °C, therefore after heat denaturing the reaction mix may be held at an optimal temperature to allow annealing of primers to the correct region of the genome based on the melting temperature (*Tm*) of the sequence to be amplified. The temperature may then be raised to around 72 °C, allowing

the polymerase enzyme to synthesise the complementary DNA (Figure 1.3). The availability of automatic thermocycling machines has also greatly facilitated the use of PCR allowing more control over the temperature cycling parameters and overcoming the tedious task of manually placing the reaction mixes in different water baths.

In order to amplify RNA, a complementary strand of DNA (cDNA) must first be produced by reverse transcription (Veres *et al.*, 1987). Using primers and dNTPs in a buffered solution, virally encoded reverse transcriptase (from e.g. Moloney murine leukemia virus or Avian myoblastoma virus) catalyses the synthesis of DNA complementary to an extracted RNA template at 37-42 °C . The primers used may be either a single specific primer to the desired sequence as shown in Figure 1.3, or a mixture of random hexanucleotide primers which allow reverse transcription of all RNA present. The product of reverse transcription is heat denatured to destroy the activity of the reverse transcriptase prior to performing PCR. The methods of reverse transcription and PCR may be adapted to quantitate specific RNA sequences.

Figure 1.3. The process of reverse transcription and PCR



With subsequent cycles, all newly synthesized DNA and original cDNA act as templates resulting in an exponential increase in PCR product

1.13 AIMS OF THE STUDY

The initial aim of this project was to study the effect of procedures used to eliminate or inactivate viral contamination of blood products such as factor VIII on HAV. This study required the development of techniques to be used in detection and quantitation of the virus. The increased use of gene amplification techniques in the field of blood virology has recently caused concern as little information is currently available in regard to the relationship between genome detection and infectivity. Therefore a further aim of this study was to investigate the use of RT-PCR in comparison with a tissue culture based infectivity assay. Since the infectivity assay relied on a highly cell culture adapted strain, the growth characteristics of this strain were studied both in tissue culture and a tamarin model and compared with that of the wild type virus and an alternative cell culture adapted strain. The use of tamarins in this study was necessary to demonstrate the changes in pathogenicity which occur upon adaptation to growth in tissue culture. Mutations which occurred during the selection of the strains used in the inactivation studies may affect the properties of the virus *in vivo* and also the stability of the virus during heat inactivation. Although not strictly within the remit of the title, the development of the PCR based quantitative assay made possible a study of the events which occur during infection of cultured cells with adapted strains of HAV and also a comprehensive comparison of the growth characteristics of the wild type and tissue culture adapted strains of the virus in a tamarin model.

Chapter 2

GENERAL METHODS

2.1 HAV GROWTH, ASSAY AND PURIFICATION

2.1.1 Virus

Wild type HAV strain HM175 was isolated during an outbreak of viral hepatitis in Australia (Daemer *et al.*, 1981) and found to cause persistent infection in a continuous African green monkey kidney cell line (BS-C-1 cells). Many variants have been described as this strain of HAV has become more adapted to growth in tissue culture. The viruses used in the present study are listed in Table 2.1

Table 2.1. Variants of HAV HM175 used in this study

HM175/wt	Wild type	Non-cytopathic
HM175/18f	Tissue culture adapted	Cytopathic in FRhK-4 cells
HM175A.2	Tissue culture adapted	Cytopathic in BSC-1 cells

HAV HM175A.2 (kindly donated by Dr D. A. Anderson, Fairfield Hospital, Victoria, Australia) was one such variant derived from wild type HM175. BS-C-1 cells persistently infected with HAV HM175/wt were subcultured 30 times followed by 8 passages of the virus at a multiplicity of infection (m.o.i.) of 0.01 radioimmunofocus forming units (RFU) / cell. A variant called HM175A was produced that was cytopathic in BS-C-1 cells. Plaque purification of this variant was carried out 3 times. A variant which produced large plaques was then selected and passaged a further 4 times as above. The resulting virus was designated HM175A.2 (Anderson, 1987).

HAV HM175/18f, (Lemon *et al.*, 1991) another variant of wild type HM175 (HM175/wt), kindly provided by Prof. S. Lemon (University of North Carolina at Chapel Hill, NC., USA) was also studied. HM175/wt was derived after 3 passages of the original isolate in marmosets. A further 3 passages in marmosets were performed followed by 16

serial passages in BS-C-1 cells. The resultant variant (HM175 p16) was capable of persistently infecting BS-C-1 cells. A cytopathic variant pHM175 was recovered after 21 to 23 subculture passages of BS-C-1 cells persistently infected with HM175 p16 at 2 to 4 week intervals. This virus was serially passaged 4 times at a low multiplicity of infection in foetal rhesus monkey kidney cells (FRhK-4) followed by 2 sequential plaque purifications in the same cells resulting in the variant HM175/18f which was used in this study.

2.1.2 Cells

Both of the cell culture adapted variants of HAV HM175, HM175A.2 and HM175/18f were propagated in BS-C-1 cells which were kindly donated by the University of Reading. Stocks of BS-C-1 cells were grown at 37 °C in Eagles Minimum Essential Medium containing 0.44 % sodium hydrogen carbonate, 15 mM HEPES pH 8.1 (BMEM) supplemented with 10 % foetal calf serum (FCS), 200 units ml^{-1} penicillin G (sodium salt), 100 units ml^{-1} streptomycin sulphate (1 % from a combined 100 x stock of penicillin and streptomycin) and 2 g ml^{-1} Fungizone (1 % from a 100 x stock). Cells were split 1:2 weekly. Confluent monolayers of BS-C-1 cells (grown in 75 cm^2 flasks, Falcon,) were washed with 50 ml PBSA which was subsequently discarded. The cells were then incubated with 20 ml 1% trypsin (Difco, 1:250) in PBSA for 5 min followed by removal of the trypsin solution and further incubation at 37 °C until the cells had detached from the surface of the flask (usually 15 min). Clumps of cells were dispersed by vigorous pipetting. A 60 ml volume of BMEM containing 10 % FCS and antibiotics as above was then added to the flask and the cells split equally between 2 fresh 75 cm^2 flasks. Cells between passages 60-80 were used for growth and assay of HAV.

2.1.3 Cytopathic microtitre plate assay of HAV HM175A.2

This method was derived from the optimisation experiments described in section 3.1.2. Virus was serially diluted in 3 fold steps in BMEM containing 1 % penicillin and streptomycin and 1 % fungizone. A 100 µl volume of each dilution was placed in appropriate wells of 96 well microtitre plates (1 dilution in each of 8 wells in a column). As a negative control, 100 µl of the above diluent was placed in each row of the first column. BS-C-1 cells grown to confluence (4-5 days) were detached by trypsinization as described previously and resuspended in the above medium containing 4 % FCS at a concentration 10^5 cells ml^{-1}. An equal volume of the cell suspension (100 µl) was added to each well of the microtitre plates which were subsequently incubated at 35 °C, 5 % carbon dioxide. After 10 days incubation 50 µl of buffered MEM containing 2 % FCS and 250 mM sodium chloride was added to each well. Plates were incubated under the same conditions for a further 2 days prior to removal of medium, followed by staining with 0.1 % Naphthalene Black in 6 % acetic acid, 166 mM NaOAc (50 µl / well) for at least 30 min and washing with tap water. Stained wells were scored positive and clear wells negative.

A score of the number of positive wells out of the number of replicates for each dilution was determined and used to estimate the titre by limiting dilution analysis based on the Poisson distribution model, using the method of maximum likelihood for "dilution assays" (Collet, 1991). This model assumes the that the probability of a positive result at a given dilution follows a Poisson distribution, and that the only factor affecting the proportion of positive results is the diluting out of the sample in the dilution series. The calculations were carried out by fitting the appropriate statistical model to the observed dilution series of numbers positive out of numbers tested, using the statistical computer program GLIM 4 (copyright 1992 Royal Statistical Society, London). The estimated end-

point is equivalent to the dilution at which there is an average of a single copy per sample tested, or the dilution at which 63% of samples tested are positive. The calculated endpoint is used to give an estimated "copies per ml" after correcting for the equivalent volume of the test sample. The values obtained were described as tissue culture infectious units (TCIU) ml^{-1}.

2.1. 4 Preparation of virus stocks

Growth medium was removed from 75 cm^2 flasks of subconfluent BS-C-1 cells and the monolayers washed with 50 ml PBSA. After removing the PBSA, cells were infected with HAV HM175A.2 or HM175/18f at a concentration of approximately 10^6 tissue culture infectious units (TCIU) ml^{-1} or RFU ml^{-1} respectively in 0.5 ml BMEM (m.o.i. = ~0.1 TCIU or RFU / cell). After 1 hr absorption at 35 °C, 30 ml serum free BMEM containing 1 % penicillin and streptomycin and 1 % fungizone was added to each flask. Flasks were then incubated at 35 °C, 5 % carbon dioxide for 9 days. Virus was released from the cells by 3 cycles of freeze thawing. Cell debris were pelleted by centrifugation at 2600 x *g*. The supernatant was extracted by mixing with an equal volume of chloroform in order to remove lipids which may be associated with the HAV capsids (Lemon and Binn, 1985), followed by centrifugation at 2600 x *g*. The aqueous phase containing virus was carefully removed and stored in 1 ml aliquots at -70 °C.

2.1.5 Purification of HAV HM175A.2

BS-C-1 cells (passage 76), split 1:2, were grown in 2 Winchester bottles (10 l) for 5 days prior to washing with PBSA and infecting with HAV HM175A.2 (m.o.i. = 0.01 TCIU / cell) as above. After 7 days incubation at 35 °C, when the majority of cells had become detached from the bottle, the contents of the Winchesters were centrifuged at 750 x *g* for 10 min to pellet the cells.

The supernatant was decanted, further clarified by centrifugation at 4800 x *g* for 30 min and mixed with polyethylene glycol and sodium chloride to final concentrations of 6.7 % and 2.3 % respectively, for 4 hr at 4 °C. The resulting mixture was then centrifuged overnight at 750 x *g*, 4 °C. The supernatant was removed and the pellet resuspended in NT buffer (100 mM sodium chloride, 10 mM Tris HCl, pH 7.4) to give a total volume of 6 ml.

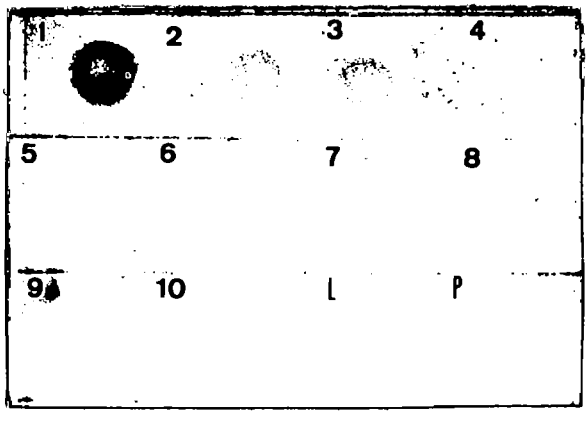
The pelleted cells were washed in 50 ml PBSA, centrifuged at 1500 rpm for 10 min and after removing the supernatant, resuspended in 6 ml NT buffer. Nonidet P-40 (10 % vol / vol) was then added to a concentration of 1 % and cells were incubated on ice for 10 min prior to centrifugation at 1000 x *g* for 10 min to remove the nuclei. The resultant supernatant was further clarified by centrifugation at 13400 x *g* for 10 min in a microcentrifuge.

Discontinuous sucrose gradients were prepared as described by Bishop *et al.* (1994). Sequential layers of 0.5 ml 80 % (vol / vol) glycerol, 100 mM sodium chloride, 100 mM Tris HCl pH 7.4, 1.8 ml each of 30 % and 20 % (wt / vol) sucrose in 100 mM sodium chloride, 10 mM Tris HCl (pH 7.4), followed by 1.8 ml of 10 % sucrose in 100 mM sodium chloride, 10 mM Tris HCl (pH 7.4), 1 % SDS were carefully layered in Beckman SW41 tubes. Gradients were overlaid with 6 ml of the above virus suspensions (virus harvested from cell and supernatant fractions were loaded onto separate gradients) and centrifuged at 37000 rpm (170000 x *g*) for 6 hr at 18 °C. The bottom of the tubes were punctured and 1 ml aliquots collected into sterile 1.5 ml microtubes.

2.1.5.1 Protein dot blot

The presence of HAV antigen in each fraction was determined by protein dot blot. During each incubation and washing step, the filter was gently agitated in the appropriate solution. From each fraction of gradient purified virus, 2 µl was spotted onto a nitrocellulose filter and allowed to air dry. The nitrocellulose filter was then blocked by gentle agitation with 5 % skimmed milk (Marvel) in PBSA for 30 min. The primary antibody, IgG affinity purified human post HAV infection convalescent serum (kindly donated by Dr Sellwood, Reading University) diluted 1:1000 in 1 % milk, PBSA, was incubated with the filter for 1 hr at room temperature. The filter was then washed twice for 10 min each wash in a total of 500 ml 1 % milk, PBSA. Goat anti-human IgG-horseradish peroxidase conjugate (Sigma Chemical Co.) was diluted 1:5000 in 1 % milk, PBSA and incubated with the filter at room temperature for 45 min. The filter was then washed as above, followed by a further wash in PBSA only for 10 min at room temperature. Substrate (0.64 mg ml^{-1} chloronaphthol in 25 mM Tris HCl pH 7.5, 0.03 % hydrogen peroxide) was then added to the filter and incubated in a light proof box for 1 hr at room temperature. The filter was then washed in PBSA for 5 min followed by a rinse in distilled water and air dried. The results of the protein dot blot showed that the majority of antigen was present in the first fraction taken from the column (Figure 2.1) as demonstrated by Bishop and co-workers (1994). This fraction was stored at -70 °C prior to use in the preparation of a working reagent for RT-PCR of HAV.

Figure 2.1 Protein dot blot of fraction from large scale purification of HAV HM175A.2



Samples

1-10 Fractions 1 to 10 from sucrose gradient

L Material loaded onto the sucrose gradient

P Positive control - HAV HM175A.2 stock.

The large scale purification of HAV was performed in order to remove unencapsidated HAV RNA from the preparation so that the RNA detected in the standards by RT-PCR would only be that extracted from virions. While the first fraction may contain a small amount of debris, naked HAV RNA would not be present in this fraction.

2.2 DETECTION OF THE HAV GENOME BY RT-PCR

Care was taken to avoid contamination of samples during RNA extraction, reverse transcription and PCR following the suggestions of Kwok and Higuchi (1989). Three separate rooms were used. The first room was used for extraction of RNA, addition to the reverse transcription mixes and incubation, and addition of cDNA to the PCR mixes. The preparation of reverse transcription and PCR mixes was performed in a separate room and amplification followed by analysis by agarose gel electrophoresis in the third room. No equipment was transferred between the rooms. Laboratory coats were changed, gloves removed and hands washed before entering a different room. At all stages, tubes were briefly centrifuged before opening to avoid release of aerosols.

2.2.1 RNA Extraction

RNA was extracted from cell culture supernatants, cells, human and tamarin sera and tamarin faeces by proteinase K / SDS extraction or using the QIAmp viral RNA extraction kit. Other established methods for total RNA extraction from clinical specimens were also attempted. These include the use of the chaotropic agent, guanidinium isothiocyanate, to release the RNA and also inactivate any ribonucleases present. The released RNA may then be purified by phenol chloroform extractions followed by ethanol or isopropanol precipitation (Chomczynski and Sacchi, 1987). Alternatively, after guanidinium isothiocyanate extraction, the purification of RNA may

be achieved by chloroform extraction and adsorption onto silica particles (Boom *et al.*, 1990, Chungue *et al.*, 1993). Use of these methods however resulted in reduced sensitivity of RNA detection by RT-PCR.

2.2.1.1 Proteinase K / SDS extraction

From each sample, 250 µl was digested at 60 °C for 30 min with 1mg ml^{-1} proteinase K and 2 % sodium dodecyl sulphate (SDS) in 50 mM Tris HCl pH 8.4, 10 mM EDTA, 200 mM sodium chloride, 40 ng ml^{-1} glycogen in a total volume of 500 µl. The digested sample was allowed to cool to room temperature prior to two phenol / chloroform extractions followed by one chloroform extraction. Extracted RNA was precipitated by adding 2.5 volumes of absolute alcohol and incubating in a solid carbon dioxide/ ethanol bath for 1 hr or -20 °C overnight. The precipitated RNA was pelleted by centrifugation at 13400 x *g* for 20 min. The resulting RNA pellet was washed twice with 1 ml 70 % ethanol. The RNA pellet was vacuum dried and redissolved in 5 µl HPLC grade H_2O.

2.2.1.2 QIAmp viral RNA Extraction Kit

The QIAmp viral RNA extraction kit (QIAGEN Ltd, Crawley, UK) was used according to the manufacturers instruction. A 140 µl sample was added to 560 µl buffer AVL containing carrier RNA (15 µg / extraction) and mixed thoroughly by vortexing. After incubation at room temperature for 10 min, 560 µl absolute alcohol was added and mixed by vortexing. Of this mixture, 630 µl was applied to a QIAmp spin column. The column was centrifuged at 6000 rpm for 1 minute in a microcentrifuge allowing the RNA extraction mix to pass through the column into a collection tube. This was repeated with the remainder of the mixture. The column was washed twice by the addition of 500 µl buffer AW and centrifugation as above. After the final wash, the column was centrifuged for a further 2 min at 13400 x *g*. The column was then dried on a heating block at 80 °C for 5 min before adding 50 µl HPLC grade water (preheated to 80 °C) and

incubating a further 5 min at 80 °C. The RNA was then eluted in to a sterile 1.5 ml microtube by centrifuging at 6000 rpm for 1 minute.

2.2.2 Reverse Transcription

RNA, purified by either proteinase K / SDS or Qiagen kit, was denatured by incubation at 75 °C for 5 min followed by quenching on ice prior to reverse transcription. The denatured RNA (5 µl) extracted and purified by either the proteinase K / SDS method or QIAmp viral RNA kit was reverse transcribed using 16 U AMV reverse transcriptase (Life Sciences Inc., St Petersburg, FL, USA) / reaction in RT buffer (50 mM Tris HCl pH8.4 (42 °C), 45 mM potassium chloride, 10 mM magnesium chloride, 4 mM DTT), containing 0.4 mM each of dATP, dCTP, dGTP and dTTP and 0.5 µg ml^{-1} random hexanucleotide primers in a final volume of 20 µl. Samples were incubated at 42 °C for 30 min followed by incubation at 80 °C for 5 min to inactivate the enzyme. The use of a ribonuclease inhibitor (RNAguard, Pharmacia Biotech) in the reverse transcription mix was found to be unnecessary. The increase in glycerol concentration in the reaction mix due to the addition of the ribonuclease reduced the efficiency of reverse transcription. Therefore ribonuclease was omitted from the reaction.

2.2.3 PCR

cDNA (5 µl) was amplified in a final volume of 50 µl containing 10 pmole of each appropriate primer, 1 x PCR buffer (20 mM ammonium sulphate, 75 mM Tris HCl, pH 9.0 (25 °C), 0.01 % (v / v) Tween), 1.5 mM magnesium chloride, 0.4 mM each of dATP, dCTP, dGTP and dTTP and 0.6 U / reaction Red Hot DNA polymerase (Advanced Biotechnologies). The PCR reaction mix was overlaid with 30 µl mineral oil. After an initial denaturation step of 94 °C for 3 min, samples were subjected to 40 cycles of 94 °C for 30 seconds, 50 °C for 20 seconds and 72 °C for 45 seconds followed by a

single cycle of 94 °C for 30 seconds, 50 °C for 20 seconds and 72 °C for 3 min using a Omnigene thermocycler (Hybaid Ltd, Teddington, UK). PCR products were analysed by running 18 μl of each sample on a 2 % agarose gel containing ethidium bromide (10 μg ml^{-1}).

Primers were designed with the aid of a computer program called Prime, part of GCG Wisconsin Sequence Analysis Package version 8 software (Genetics Computer Group, Madison, WI, USA). This programme checks primers for self-complementarity and pairs of primers for complementarity to each other. A further test is also used to determine complementarity between the primer and any non-specific binding sites on the template cDNA. By default, the programme requires G or C as the 3'-terminal base of all selected primers. Since G and C base pair via 3 hydrogen bonds, the presence of these bases at the 3' end of the primer is less likely to result in mis-matching than A or T which pair with only 2 hydrogen bonds.

PCR primers - VP1/2a Junction (699 bases)

Forward	A7a	5' GGTCTTGCCGTTGATACTCC 3' 2694 2713
Reverse	A8a	5' GAACCCCAGCATCCATTTC 3' 3393 3375

The VP1/2a junction region of the HAV genome was chosen for amplification as this site contains a variable region which may be sequenced to determine the genotype of any unknown HAV isolate. Primers were selected to flank this variable region so that they would amplify all known genotypes. Initially, primers previously designed by Robertson and co-workers (1992) which recognise the VP1/2a junction were synthesised and used.

However, the levels of sensitivity of PCR amplification using these primers was not acceptable for this study.

2.2.4 Preparation of working reagents for standardisation of RNA extraction and RT-PCR assays

HAV working reagents were prepared in plasma cryosupernatant (CS101) and faecal material (FS232) so that the extraction of RNA from such samples and RT-PCR could standardised. The plasma cryosupernatant was kindly donated by Dr Harrison (Bio Products Laboratories, Elstree, UK). For the faecal working reagent, a 10 % suspension of tamarin faeces was prepared in BMEM and clarified by centrifugation at 1300 x *g* for 10 min. HAV strain HM175A.2 (7.7 $\log_{10}$ TCIU ml^{-1}), prepared from tissue culture by large scale purification, was diluted in 10 fold steps to a $1:10^3$ dilution in the cryosupernatant and faecal material. The resulting working reagents were aliquoted in 140 µl volumes and stored at -20 °C. The cryosupernatant standard is always PCR positive when assayed at a 1:10 and positive 80 % of the time at a 1:100 dilution. The standard was included in each quantitative assay in duplicate at both a 1:10 and 1:100 dilution. PCR results were only accepted if both of the 1:10 samples and 1 of the 1:100 samples were positive. The 1:10 dilution is expected to contain 3.7 $\log_{10}$ TCIU ml^{-1} and the 1:100 dilution, 2.7 $\log_{10}$ TCIU ml^{-1}. Since only 3.5 µl of the diluted standard is present in each PCR mix the amount of RNA from infectious virus present in each reaction should be 20 TCIU and 2 TCIU respectively. The plasma cryosupernatant standard, CS101, was included in each extraction run when assaying any blood product or tissue culture sample. The faecal standard, FS232, was used in the same way as the cryosupernatant standard when tamarin stool samples were tested. The sensitivity of detection of the faecal standard was the same as that of the cryosupernatant standard. The production of standards was done in a room not previously used for HAV growth, genome extraction or amplification using the precautions mentioned above.

2.2.5 PCR quantitation by end point dilution

Extracted HAV RNA was quantitated by an end point dilution method. Initially, a sample of extracted RNA was serially diluted in 3 fold steps in HPLC grade water and reverse transcribed as above. The lowest dilution of the resulting cDNA was also serially diluted in 3 fold steps. Five replicates of each dilution (made at both the RNA and cDNA stages) were amplified as described above. Analysis of the results (Figure 2.2) showed that titres estimated by dilution of cDNA (6.05 $\log_{10}$ genomes ml^{-1}) were comparable to those obtained by dilution of RNA (6.04 $\log_{10}$ genomes ml^{-1}).

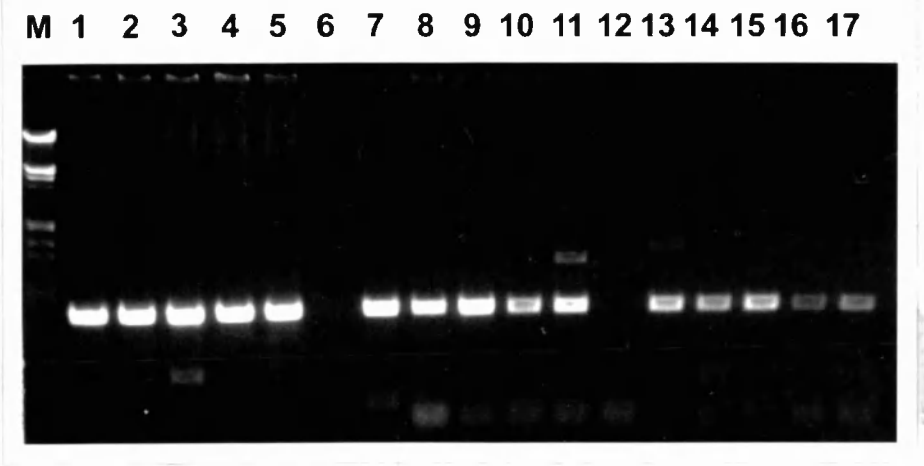
In all subsequent quantitative PCR assays, serial dilutions of cDNA were made. In order to determine an approximate concentration of HAV RNA in samples prior to quantitation, serial 1:10 dilutions of the cDNA were first amplified. Five replicates of 3 fold serial dilutions were then made from the concentration of the penultimate positive dilution. A score of the number of PCR positive samples out of the number of replicates for each dilution was determined and used in analysis with the log dilution to estimate the titre of HAV RNA using the computer program GLIM 4 as in the cytopathic microtitre plate assay. This value was adjusted to give a titre in $\log_{10}$ genome equivalents (genomes) ml^{-1} as only 3.5 µl of original sample was present in the final PCR reaction mix.

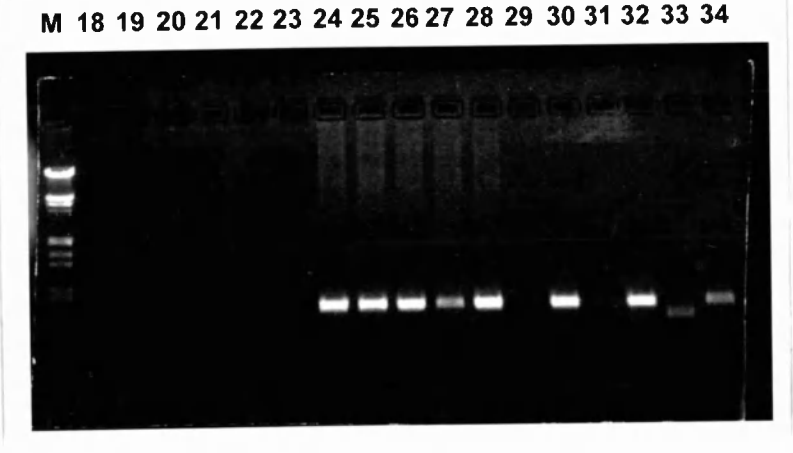
As there was a decrease in band intensity in the higher PCR positive dilutions, it could not be assumed that the endpoint was digital. A gradual fading of band intensity at the higher dilutions until it was no longer possible to visualise bands by ethidium bromide staining may have occurred. However, nested PCR showed that no further bands could be detected and that single round PCR was adequate for digital enpoint detection (see appendix A).

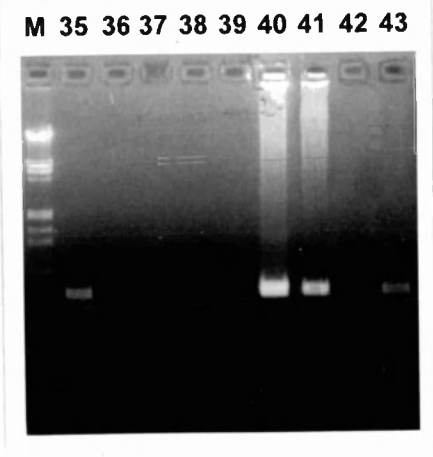
Figure 2.2 Comparison of RNA and cDNA PCR titration

Samples

M	Molecular weight markers 1
1-5	Replicates of RNA diluted $1:10^{1.5}$ and reverse transcribed
6	Negative control water
7-11	Replicates of RNA diluted $1:10^{2}$ and reverse transcribed
12	Negative control water
13-17	Replicates of RNA diluted $1:10^{2.5}$ and reverse transcribed
18-22	Replicates of RNA diluted $1:10^{3}$ and reverse transcribed
23	Negative control water
24-28	Replicates of cDNA diluted $1:10^{2}$
29	Negative control water
30-34	Replicates of cDNA diluted $1:10^{2.5}$
35-39	Replicates of cDNA diluted $1:10^{3}$
40-41	Standard CS101 1:10
42-43	Standard CS101 1:100


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17


M 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34


M 35 36 37 38 39 40 41 42 43

Chapter 3

OPTIMISATION AND VALIDATION OF METHODS

3.1 OPTIMISATION OF THE CYTOPATHIC MICROTITRE PLATE ASSAY

3.1.1 Introduction

Prior to adaptation of the virus to growth in tissue culture, HAV antigen and antibodies were quantitated by various radioimmunoassays (Hollinger *et al*, 1975, Purcell *et al*, 1976). Subsequent methods developed to assay HAV in tissue culture include a radioimmunofocus assay (RIFA) (Lemon *et al.*, 1983), *in situ* radioimmunoassay (RIA), and a fluorescent focus assay (Siegl *et al.*, 1984). Each of these assays is complex and time consuming, involving the growth of HAV in tissue culture for at least a week followed by the use of labelled antibodies to detect foci or relative quantity of the virus.

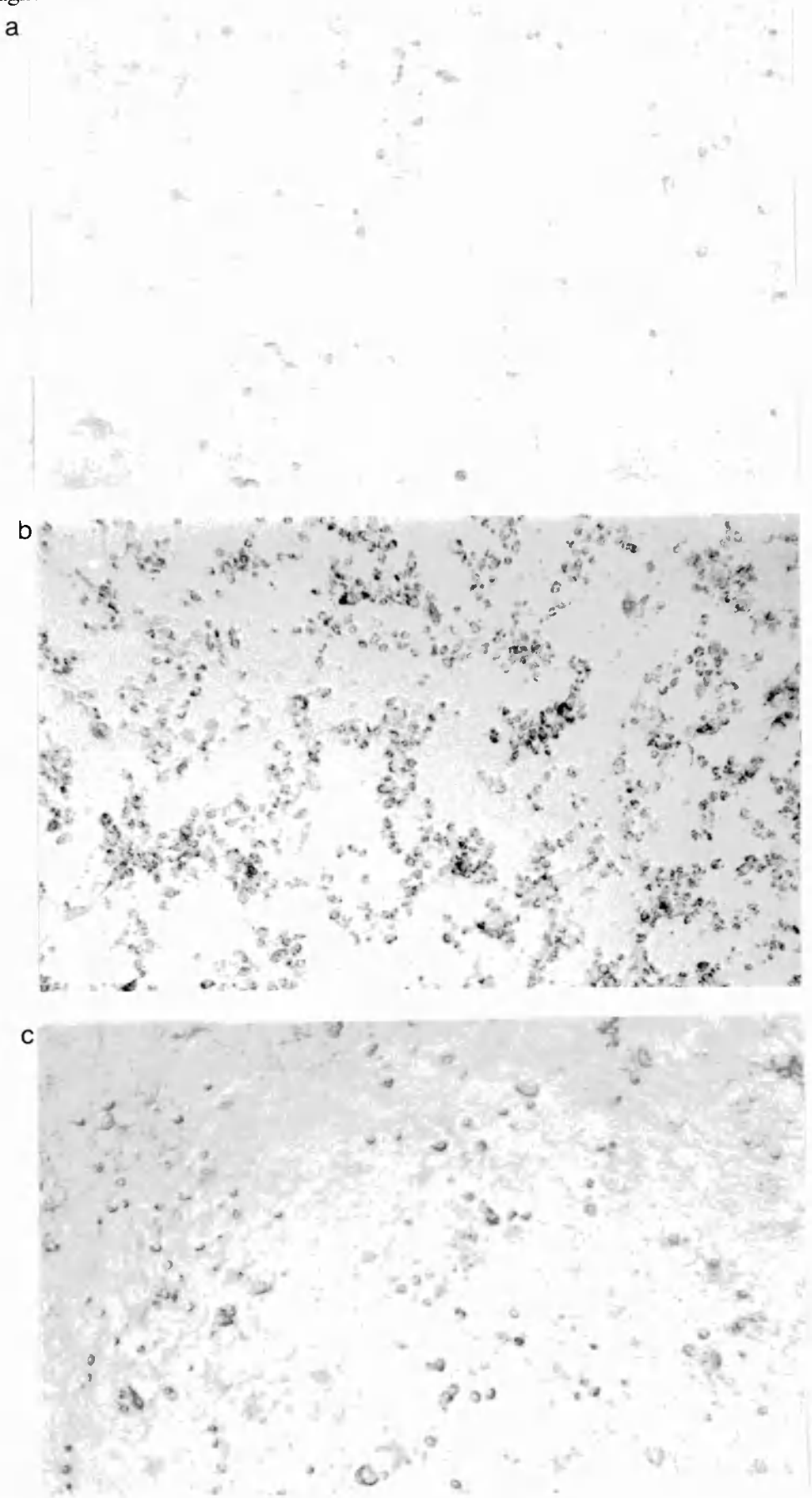
Various assays have been developed to measure virus titres in 50 % infectious doses ml^{-1} ($TCID_{50}$). Again, these are complex as they rely on either enzyme immunoassay (Yap and Lam, 1994), fluorescent antibody detection and microscopic analysis (Nadala and Loh, 1990) or adsorbing, washing and eluting radio-iodinated antibodies from individual wells prior to scintillation analysis (Krah, 1991).

Recently, cytopathic strains of the virus have been isolated and plaque purified for use in cytopathic plaque assay (Anderson, 1987, Cromeans *et al.*, 1987). Initial attempts to use this assay for HAV were difficult to reproduce due to problems in maintaining the integrity of cell monolayers for the 2 week incubations described. Therefore a cytopathic microtitre plate assay which does not require the use of an agarose overlay was developed and routinely used.

3.1.2 Results

Infection of BS-C-1 cells with HAV HM175A.2 resulted in the appearance of cytopathology as shown in Figure 3.1. Little cytopathology was seen in these cells

Figure 3.1 Cytopathology of HAV HM175A.2 and HM175/18f in BS-C-1 cells
Photographs of unstained cells taken at 12 days post infection: a) Negative control mock infected BS-C-1 cells, b) HM175A.2 infected BS-C-1 cells showing rounding, clumping and detachment of ~ 95% of cells and c) HM175/18f infected BS-C-1 cells showing only rounding of occasional cells. Magnification x30.



when infected with HAV HM175/18f. BSC-1 cell monolayers were infected as described (2.1.4 Preparation of virus stocks) with 10^3 TCIU HAV HM175A.2 or 10^3 RFU HAV HM175/18f. Cytopathology, characterised by rounding, detachment and aggregation of approximately 95% of cells was seen in HAV HM175A.2 infected cells by 10-14 days post infection. HAV HM175A.2 was subsequently used to develop a cytopathic microtitre plate assay.

The method described in Chapter 2 was chosen after careful optimisation of conditions. To determine the optimum concentration of cells, repeated assay of HM175A.2 Preparation 13 was performed using cells at varying concentrations from 10^3 cells / well to 10^5 cells / well. The optimum number of BS-C-1 cells was found to be 10^4 cells / well. If more than $10^{4.5}$ cells were added to each well, the cells quickly over grew and began to die and with less than $10^{3.5}$ cells / well, the staining of negative control wells was less distinct (Figure 3.2 and Table 3.1). Attempts to assay the virus by infecting monolayers of BS-C-1 cells previously seeded onto the microtitre plates were unsuccessful. Mixing of cells with virus at room temperature or 37 °C for 1 hr before addition to wells made no difference to the results and since this technique was more cumbersome, it was not adopted routinely. Although attachment of HAV occurs most efficiently with serum-free medium (Zajac *et al*, 1991), the BS-C-1 cells grew poorly and the addition of 2 % FCS was found to be optimum (Figure 3.3, Table 3.2). The use of a synthetic serum substitute (Clex, Dextran Products Ltd) was also investigated. When 2 % Clex was added to the medium, the results obtained were no better than those observed with 2 % FCS. The addition of sodium or magnesium ions to the growth medium to enhance HAV cytopathology has been reported (Anderson, 1987, Cromeans *et al.*, 1987). In this assay, the effect of various concentrations of sodium chloride or magnesium chloride was investigated. No improvement of cytopathology was achieved by the addition of 26 mM

magnesium chloride and the general appearance of uninfected cells was impaired. The addition of 100 mM sodium chloride after 10 days infection improved the clarity of the CPE without damaging the uninfected cells. Increasing the concentration of sodium chloride to 200 mM led to general cell death (Figure 3.4 and Table 3.2).

A cell incubation temperature of 35 °C was chosen after comparison with incubation at 37 °C and 34 °C. The range of incubation temperatures tested are those previously cited for growth and assay of cytopathic strains of HAV (Anderson, 1987, Cromeans *et al.*, 1987, Nasser and Metcalf, 1987 and Lemon *et al.*, 1991).

Table 3.1. Optimisation of cell concentration

No. of cells /well	Appearance of control cells after 1 week	Appearance of stained control cells
$10^{3.0}$	incomplete monolayers	faint, patchy staining
$10^{3.5}$	good	faint staining
$10^{4.0}$	**good**	**good dark staining**
$10^{4.5}$	good	patchy dark staining
$10^{5.0}$	cells piled up and died	dead cells detached

Table 3.2. Optimisation of assay conditions

Concentration of FCS in wells	Appearance of stained uninfected wells	Appearance of stained infected wells
0%	cells did not survive	cells did not survive
1%	faint, patchy staining	very little stain
2%	**good dark staining**	**very little stain**
2.5%	patchy dark staining	patchy dark staining
Concentration of NaCl in wells*		
50mM	good dark staining	faint patchy staining
100mM	**good dark staining**	**very little stain**
150mM	faint, patchy staining	very little stain
200mM	faint, patchy staining	very little stain

* Added after 10 days incubation

Figure 3.2 Effect of cell concentration on the cytopathic microtitre plate assay of HAV HM175A.2

HM175A.2 preparation 13 assayed in BS-C-1 cells. Column 1, uninfected cells; column 2, HM175A.2 $1:10^2$ dilution; columns 3-12, 0.5 log dilutions of HM175A.2 starting at $1:10^3$. BS-C-1 cell concentrations a) $10^{3.5}$ cells / well and b) $10^{4.5}$ cells / well.

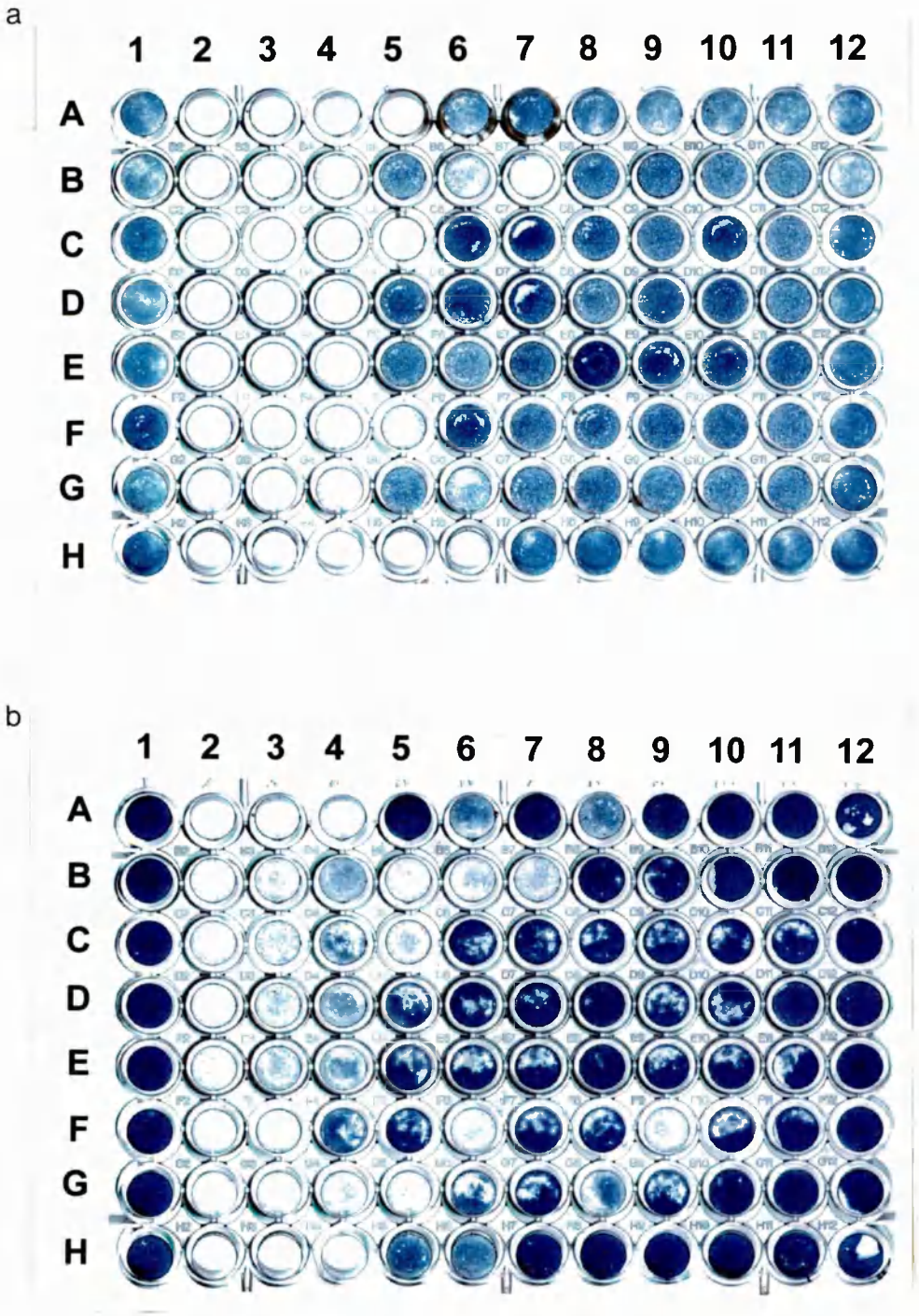


Figure 3.3 Effect of FCS concentration on the cytopathic microtitre plate assay of HAV HM175A.2

HM175A.2 preparation 13 assayed in BS-C-1 cells. Column 1, uninfected cells; column 2, HM175A.2 $1:10^2$ dilution; columns 3-12, 0.5 log dilutions of HM175A.2 starting at $1:10^3$. FCS concentrations a) 1 % and b) 2.5 %.

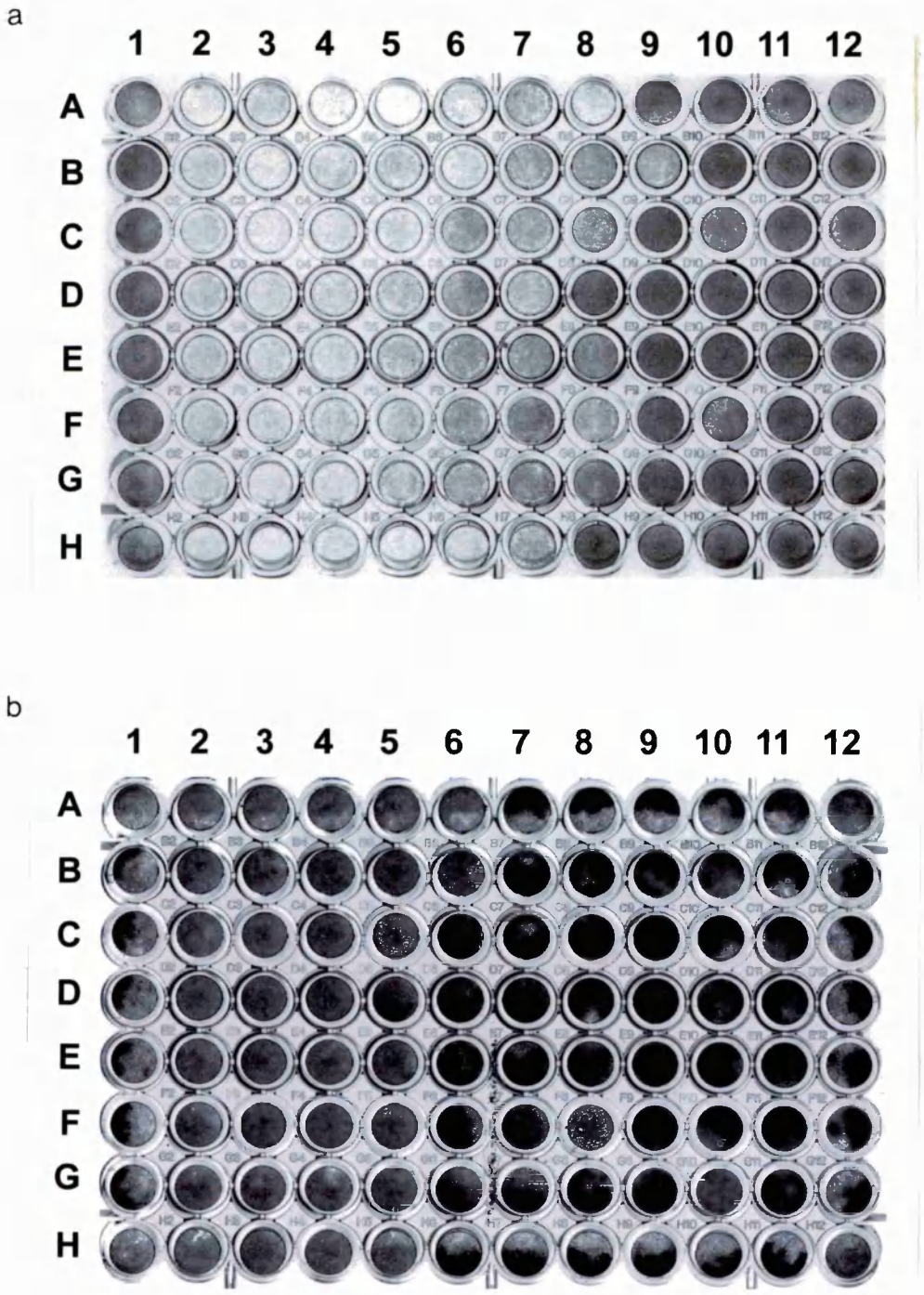
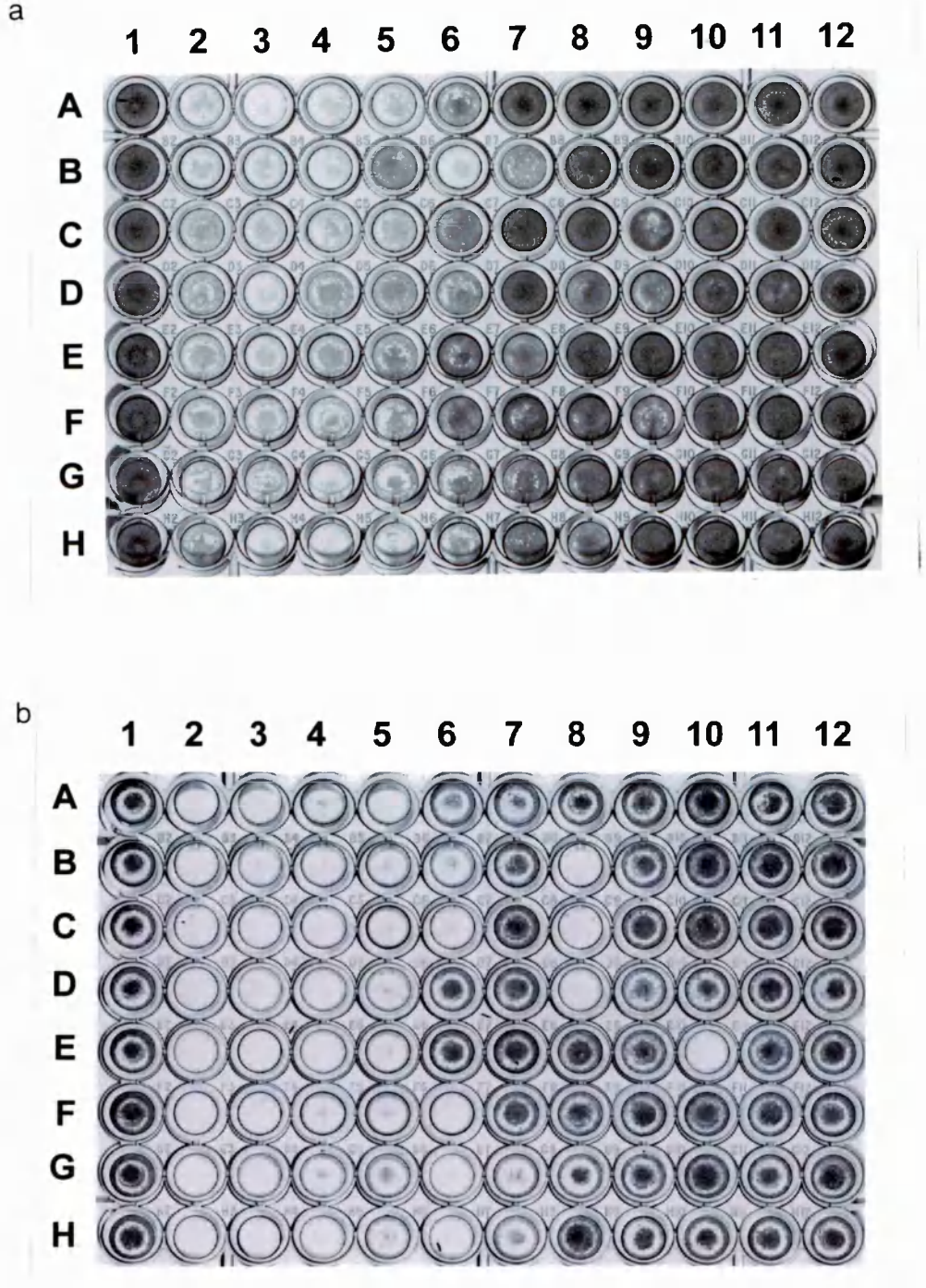


Figure 3.4 Effect of sodium chloride concentration on the cytopathic microtitre plate assay of HAV HM175A.2

HM175A.2 preparation 13 assayed in BS-C-1 cells. Column 1, uninfected cells; column 2, HM175A.2 $1:10^2$ dilution; columns 3-12, 0.5 log dilutions of HM175A.2 starting at $1:10^3$. Concentration of sodium chloride added at 10 days post infection a) 50 mM and b) 150 mM



Using the optimised conditions, a good CPE was obtained in infected cells. The titre of the tissue culture adapted strain of HAV, HM175A.2, was easily determined using this assay. A representative assay plate is shown in Figure 3.5 and the scoring in Table 3.3. The log TCIU was determined by GLIM analysis to be 4.71. As only 100 µl of diluted virus was placed in each well, this figure was adjusted by a factor of 10 to give a titre of 5.71 log TCIU ml^{-1}.

Assay plates were evaluated twice independently by blind microscopic analysis prior to staining. There was no person to person variation in microscopic evaluation. Scores obtained by microscopy and viewing stained plates by eye were exactly the same.

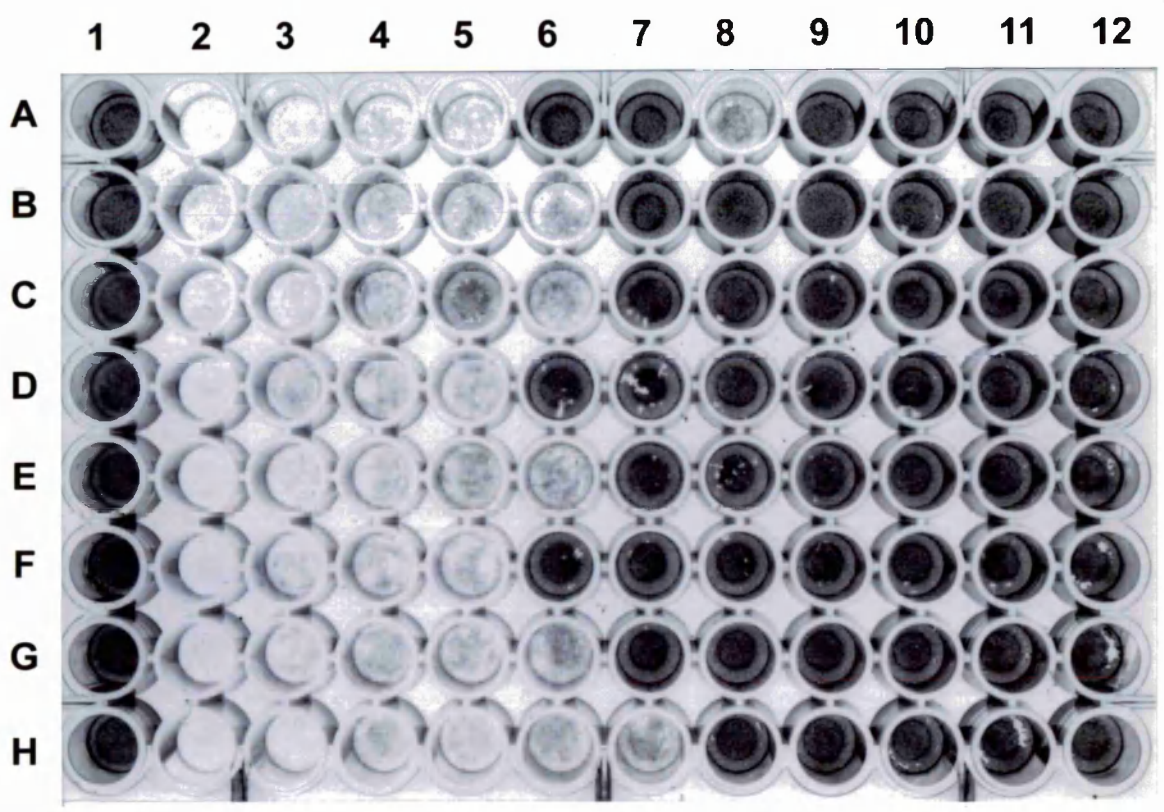


Figure 3.5 Cytopathic microtitre plate assay of HM175A.2 preparation 13
Wells in column 1 contain negative control uninfected BS-C-1 cells. Column 2, HM175A.2 $1:10^2$ dilution; columns 3-12, 0.5 log dilutions of HM175A.2 starting at $1:10^2$

Table 3.3. Example of endpoint titration

log_{10} dilution	wells infected	wells uninfected	infected / replicates	
			Ratio	%
-2.0	8	0	8/8	100
-3.0	8	0	8/8	100
-3.5	8	0	8/8	100
-4.0	8	0	8/8	100
-4.5	5	3	5/8	62.5
-5.0	1	7	1/8	12.5
-5.5	1	7	1/8	12.5
-6.0	0	8	0/8	0
-6.5	0	8	0/8	0
-7.0	0	8	0/8	0
-7.5	0	8	0/8	0

This cytopathic microtitre plate assay was also attempted using the HAV strain HM175/18F. CPE was noted at 12 days post infection when plates were evaluated by microscopic analysis, but the extent of cytopathology was not sufficient to determine the titre of the virus after staining (Figure 3.6). The reduced cytopathology of HM175/18f in BS-C-1 cells may be due to the fact that this variant had been clonally selected in FRhK-4 cells. The mutations required for cytopathic growth of HAV in this cell line may differ from those producing cytopathology in BS-C-1 cells.

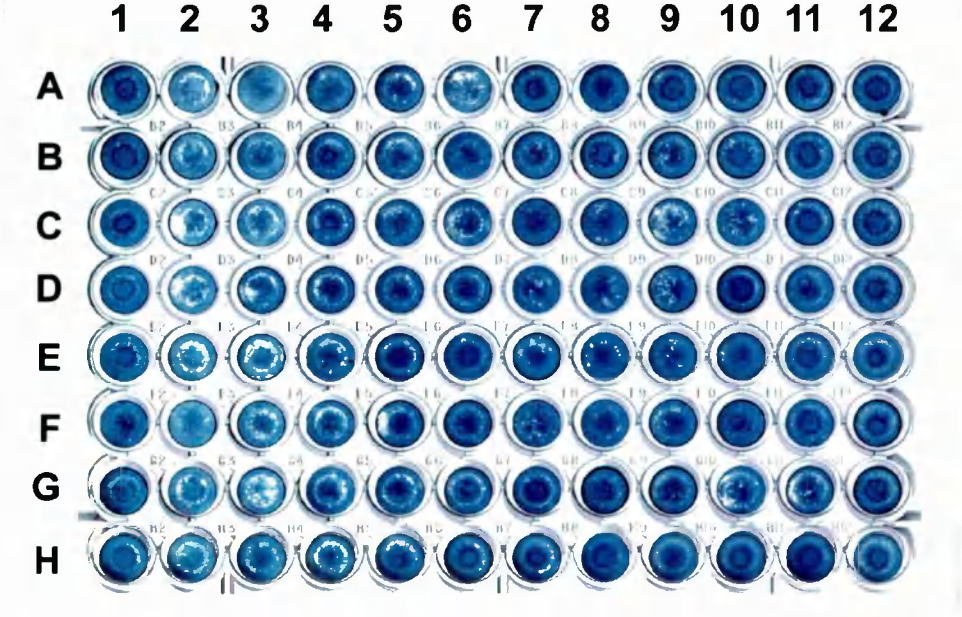


Figure 3.6 Cytopathic microtitre plate assay of HAV HM175/18f
Tissue culture extracted HM175/18f assayed in BS-C-1 cells. Column 1, uninfected cells; column 2, HM175/18f $1:10^3$ dilution; columns 3-12, 0.5 log dilutions of HM175/18f starting at $1:10^2$.

3.1.1.2 Analysis of Assay Repeatability

The virus stock HM175A.2-13 was assayed by the optimised conditions a further ten times (Table 3.4) so that the statistical reproducibility of this cytopathic assay could be determined. Variability of the assay when repeated on the same day was calculated and expressed as a percentage coefficient of variation (% CV). The % CV is the standard deviation represented as a percentage of the mean. Similarly, analysis was also performed to determine the day to day variability (Table 3.5). The overall coefficient of variation between assays was found to be 4.0 %.

Table 3.4. Repeat assays of HAV HM175A.2 preparation 13

Assay number	Assay day	$\log_{10}$ TCIU ml^{-1}
1	28/3	5.56
2	13/4	5.68
3	13/4	5.78
4	13/4	5.81
5	20/7	5.71
6	20/7	5.71
7	20/7	5.82
8	29/7	5.75
9	4/8	5.19
10	4/8	5.28

Table 3.5. Variation between assays

Variation	coefficient of variation
Within-day	1.4%
Day to day	4.1%
Overall	3.97%

3.1.3 Antibody neutralisation assay

In order to demonstrate that the cytopathology obtained in the microtitre plate assay was due to infection of BS-C-1 cells with HAV, several antibody preparations were assayed against a low titre of HAV HM175A.2, showing the ability of antisera to HAV to neutralise the virus.

Antibody preparations or immune sera were serially diluted in 10 fold steps in serum-free BMEM. Cytopathic HAV strain HM175A.2 was diluted to $10^{3.5}$ TCIU ml^{-1} in serum-free buffered MEM. This concentration of virus was found to be the minimum titre required to give CPE in all wells. Equal volumes of the various antibody dilutions and virus were continuously mixed on a shaker for 2 hr at room temperature. Of each antibody dilution / virus mix, 20 µl were placed in appropriate wells of 96 well microtitre plates (1 dilution in each of 8 wells in a column). . In wells of the first column 20 µl of diluent only was placed as cell control and in the second column, 20 µl of an equal mix of medium and diluted virus as a virus control. BS-C-1 cells cultured as for cytopathic assay were resuspended at a concentration of $10^{4.5}$ ml^{-1} in buffered MEM containing 2 % FCS. The cell suspension (200µl) was added to each well of the microtitre plates which were subsequently incubated at 35 °C, 5 % carbon dioxide and treated exactly as in the above cytopathic assay. The titre of virus used was sufficient to cause cell destruction in all of the virus control wells. However, if specific antibody was present, the CPE was inhibited by neutralisation of the virus. Therefore, if the cell monolayer remained intact, the well was scored positive for antibody. The results were analysed using the GLIM 4 statistical computer program to give titres of cytopathology neutralising units ml^{-1}.

The WHO international anti-HAV human immunoglobulin standard (Gerety, 1983) was capable of neutralising the CPE of the virus in this assay system. Figure 3.7 shows the

results of one neutralisation assay using the WHO standard. The titre determined by GLIM analysis of this assay of the WHO standard was 1:182000. Pooled immune human sera preparations and mouse sera from vaccine potency tests were also able to neutralise the virus (Figure 3.8). Non -immune human serum and pre-inoculation mouse sera did not appear have any neutralising affect on the virus.

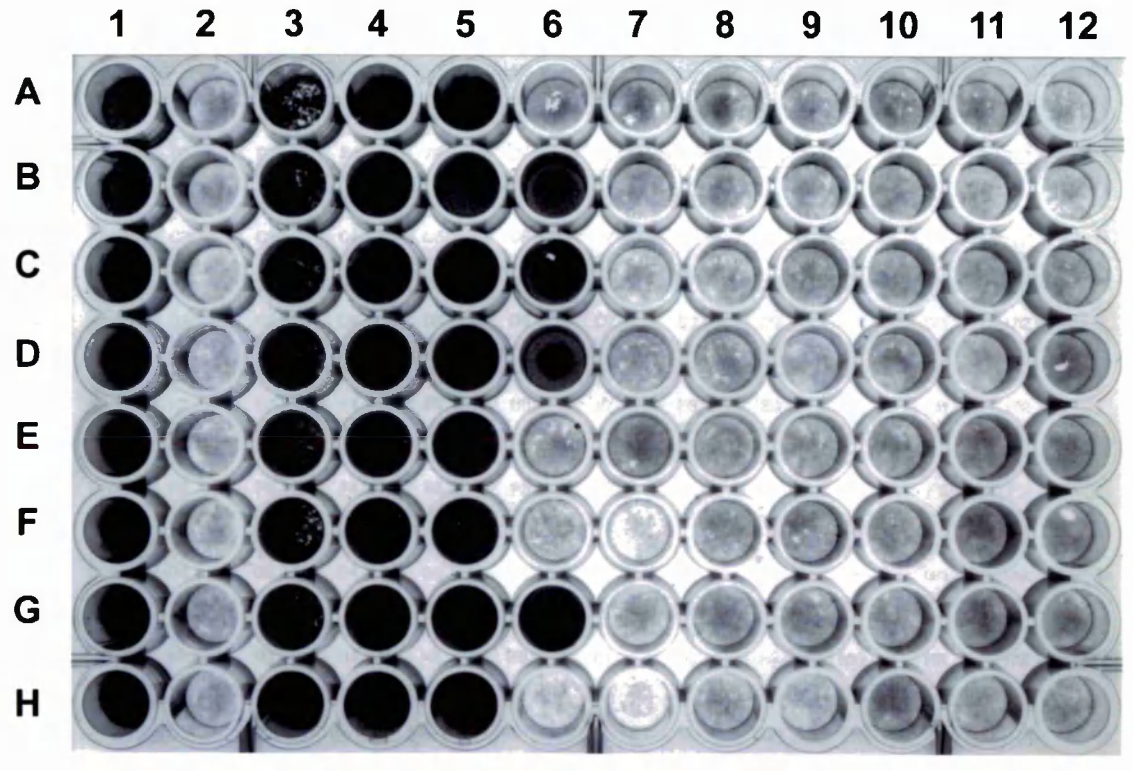


Figure 3.7 Assay of WHO immune serum globulin standard for HAV neutralising antibodies

The wells in column 1 contain uninfected BS-C-1 cells. The cells in the second column were infected with $10^{1.5}$ TCIU / well HAV HM175A.2. Subsequent columns (3-12) were inoculated with virus neutralised by serial 10 fold dilutions of the WHO standard starting from a $1:10^2$ dilution (1IU/ml).

Neutralising antibody titres could be quantified by incubation of a known challenge dose of HAV HM175A.2 with serial dilutions of immune sera or other antibody preparations. The residual infectious virus was detected by standard cytopathic assay. The end point titre of the WHO standard anti-HAV determined by this neutralisation assay was consistently 100 fold higher than those obtained by commercial HAV antibody detection

kits. The lower limit of detection of anti-HAV in the described antibody neutralisation assay is approximately 1 mIU ml^{-1}.

An adaptation of RIFA called the radioimmunofocus inhibition test (RIFIT) is commonly used to detect neutralising antibodies. In this assay, a known quantity of tissue culture adapted virus is incubated with the test serum prior to performing the RIFA. The RIFIT

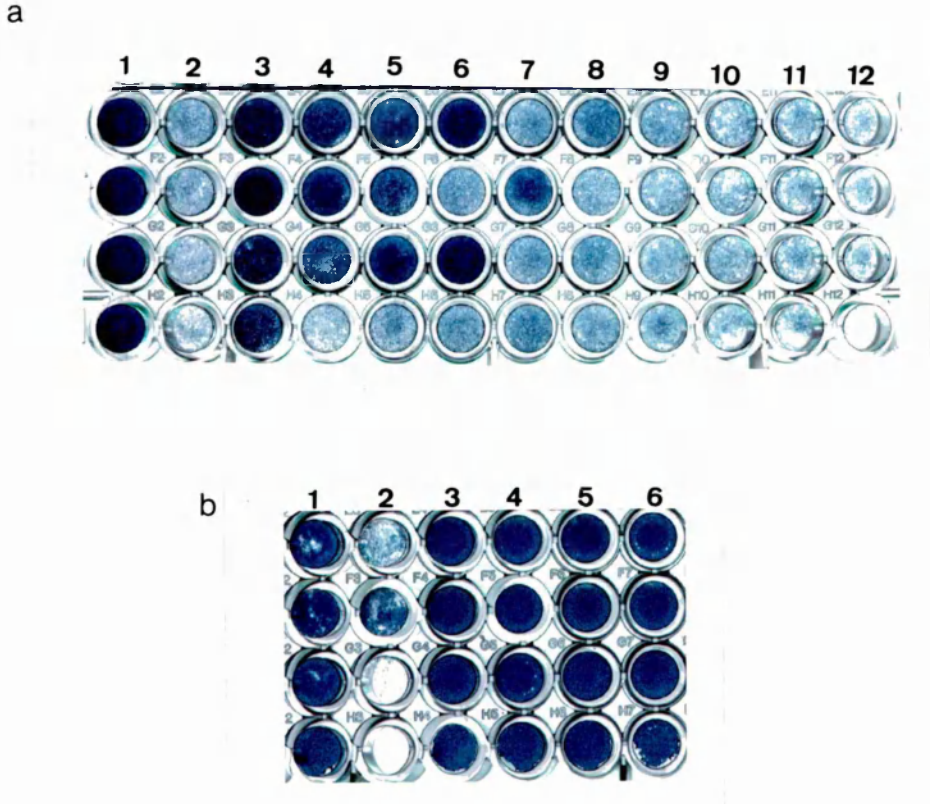


Figure 3.8 Neutralisation of HAV HM175A.2 cytopathology by pooled human sera and mouse sera from vaccine potency tests

a) Pooled human post convalescent sera (supplied by Reading University). Column 1, uninfected BS-C-1 cells; column 2, virus control ($10^{1.5}$ TCIU / well); columns 3-12, Pooled sera diluted 1:10 in BMEM followed by serial 3 fold dilutions.
b) Assay of mouse sera from vaccine potency test (10µl at a 1:10 dilution in BMEM / well). Column 1, uninfected BS-C-1 cells; column 2 mock inoculated mouse serum; Columns 3-6, sera from mice inoculated with dilutions of 'HAVRIX' (SmithKline Beecham) - Column 3, 1:24; Column 4, 1:48; Column 5, 1/96; Column 6, 1/192

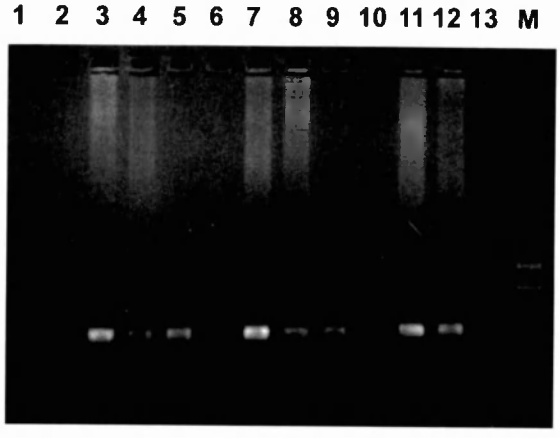
has the same disadvantages as are encountered with the RIFA. A similar phenomenon was observed when the WHO international immune serum globulin standard was assayed by the RIFIT. Using this method titres between 1:150000 and 1:800000 were obtained in various different laboratories (Stapleton *et al*, 1985, Wood, unpublished data). This HAV cytopathic inhibition assay is less labour intensive and simpler to perform than the RIFIT.

3.2 OPTIMISATION OF HAV RT-PCR

3.2.1 Comparison of proteinase K / SDS and QIAmp kit methods in HAV RNA extraction

The proteinase K / SDS method was successfully used to extract HAV RNA from tissue culture, serum and blood product samples. However, the results of spiking experiments showed that this method was not suitable for routine detection of HAV RNA in stool samples. A reduction in sensitivity of the RT-PCR method when testing the HAV spiked stool samples was probably due to inefficient removal of contaminants which inhibited the reverse transcription and PCR. Using the QIAmp viral RNA extraction kit (Qiagen Inc.), the sample is incubated with a chaotropic agent, ethanol precipitated and centrifuged through a silica membrane. The membrane bound RNA may then be washed several times before elution in water. RT-PCR of HAV RNA extracted from tamarin stool samples using the QIAmp extraction kit was successful. The two methods were subsequently compared for water, normal tamarin serum and stool samples spiked with tissue culture extracted HM175A.2 at concentrations of 10^5 TCIU ml^{-1} (~ 14000 TCIU / extraction) and 10^3 TCIU ml^{-1} (~140 TCIU / extraction). A 10 % suspension of the tamarin stool sample in BMEM was prepared and clarified by centrifugation as previously described, prior to the addition of the virus. Using both

Figure 3.9 Comparison of Proteinase K / SDS RNA extraction with the QIAmp viral RNA kit



Samples

1 HAV spiked faecal suspension 10^5 TCIU ml^{-1} (Proteinase K/SDS)

2 HAV spiked faecal suspension 10^3 TCIU ml^{-1} (Proteinase K/SDS)

3 HAV spiked faecal suspension 10^5 TCIU ml^{-1} (QIAmp kit)

4 HAV spiked faecal suspension 10^3 TCIU ml^{-1} (QIAmp kit)

5 HAV spiked tamarin serum 10^5 TCIU ml^{-1} (Proteinase K/SDS)

6 HAV spiked tamarin serum 10^3 TCIU ml^{-1} (Proteinase K/SDS)

7 HAV spiked tamarin serum 10^5 TCIU ml^{-1} (QIAmp kit)

8 HAV spiked tamarin serum 10^3 TCIU ml^{-1} (QIAmp kit)

9 HAV spiked water 10^5 TCIU ml^{-1} (Proteinase K/SDS)

10 HAV spiked water 10^3 TCIU ml^{-1} (Proteinase K/SDS)

11 HAV spiked water 10^5 TCIU ml^{-1} (QIAmp kit)

12 HAV spiked water 10^3 TCIU ml^{-1} (QIAmp kit)

13 Negative control water

M Molecular weight markers 2

methods, 140 µl volumes of were extracted and the purified RNA resuspended or eluted in 50 µl water. Of the extracted RNA, 5 µl was reverse transcribed in 20 µl reaction mixes as described previously and 5 µl of the resulting cDNA was amplified by PCR, using primers HA1F and HA1R (See appendix B).

The proteinase K / SDS extraction method allowed amplification of HAV HM175A.2 in water and serum at both the high and low concentrations (Figure 3.9). However no HAV RNA was detected in the proteinase K/SDS extracted tamarin stool samples. HAV RNA was detected by RT-PCR in all spiked samples which had been extracted by the QIAmp kit. This method was subsequently used in all RNA extractions prior to RT-PCR

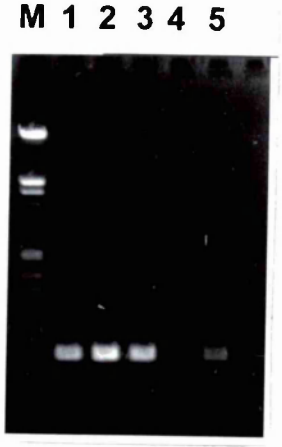
3.2.2 Optimisation of PCR conditions

3.2.2.1 Magnesium chloride titration

In order to determine the optimal magnesium chloride concentration required for PCR using primers A7a and A8a, cDNA reverse transcribed from tissue culture extracted HAV RNA was amplified in reaction mixes containing differing concentrations of the salt. Each reaction mix contained 1 x PCR buffer, 0.2 mM each of dATP, dCTP, dGTP and dTTP and 0.4 µM primers A7a and A8a. Magnesium chloride was added to individual reactions at concentrations of 1 mM, 1.5 mM, 2 mM, 2.5 mM and 3 mM. The cDNA was diluted 1:10 in reverse transcription buffer and 5 µl added to each PCR reaction mix. The tubes were subjected to 30 amplification cycles indicated above and 15 µl of the amplification products were analysed on a 1 % ethidium bromide-agarose gel. The optimal conditions were those which gave the greatest product band intensity as determined by eye. Figure 3.10 shows that the optimal magnesium chloride concentration for PCR using primers A7a and A8a is 1.5 mM.

Figure 3.10 Optimisation of RT-PCR for HAV RNA

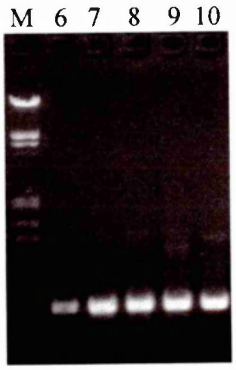
a) Magnesium chloride titration



Samples

M	molecular weight markers 1
1	1 mM magnesium chloride
2	1.5 mM magnesium chloride
3	2 mM magnesium chloride
4	2.5 mM magnesium chloride
5	3 mM magnesium chloride

b) PCR primer titration



M	molecular weight markers 1
6	0.1 μM primers
7	0.2 μM primers
8	0.4 μM primers
9	0.8 μM primers
10	1 μM primers

c) Red hot polymerase titration

M	molecular weight markers 1
11	0.2 U Red hot polymerase
12	0.4 U Red hot polymerase
13	0.6 U Red hot polymerase
14	0.8 U Red hot polymerase
15	1 U Red hot polymerase

3.2.2.2 Primer titration

The above cDNA preparation was also used in the titration of primers A7a and A8a. Each reaction mix contained 1 x PCR buffer, 1.5 mM magnesium chloride and 0.2 mM each of dATP, dCTP, dGTP and dTTP. Primers were added to individual reactions at concentrations of 0.1 μM, 0.2 μM, 0.4 μM, 0.8 μM and 1 μM. The cDNA was added and tubes were treated exactly as above. The optimum concentration of primers was found to be 0.2 μM. While higher concentrations of primer also gave comparable results, the above concentration was chosen in order to conserve resources.

3.2.2.3 Red Hot polymerase titration

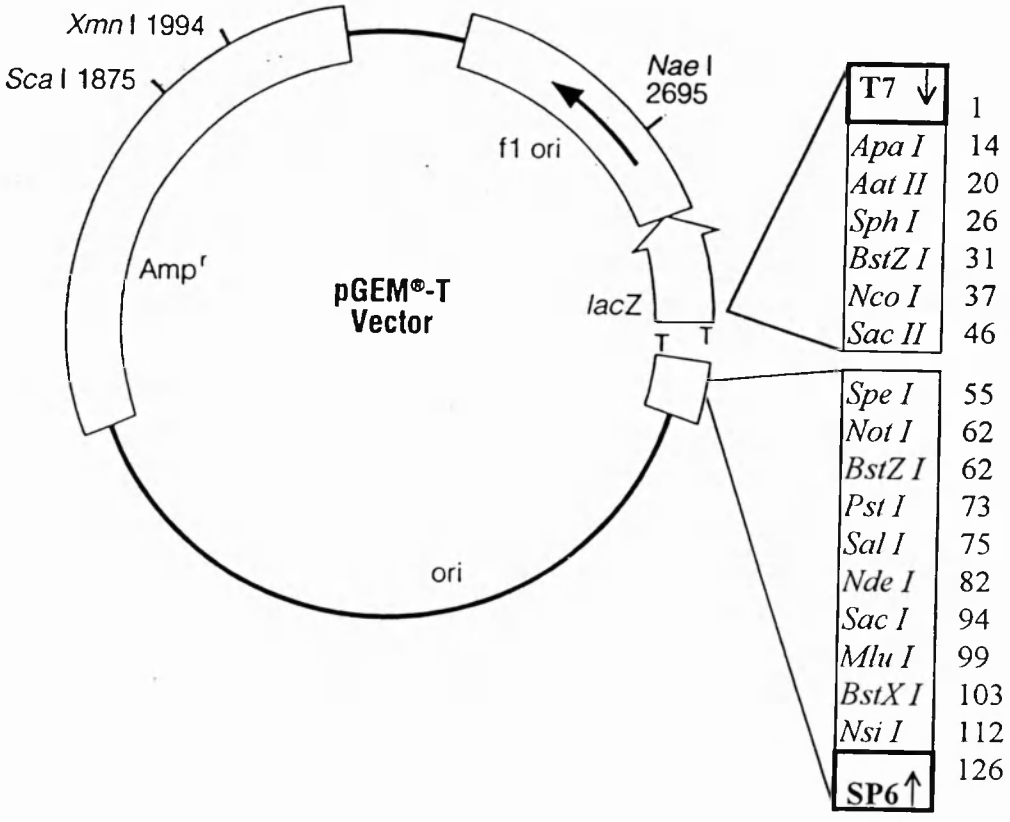
The amount of Red Hot polymerase required for optimal amplification of HAV RNA with primers A7a and A8a was determined. Red Hot polymerase was added at 0.2 - 1 unit / reaction in 0.2 unit increments to 50 μl reaction mixes containing 1x PCR buffer, 1.5 mM magnesium chloride, 0.2 mM each of dATP, dCTP, dGTP and dTTP, primers A7a and A8a (0.2 μM), and 5 μl of the above control cDNA. Tubes were subjected to 30 amplification cycles and analysed as above. The PCR product bands became more intense with increasing Red hot polymerase concentration until 0.6 units / reaction. As no further improvement was seen, this concentration of enzyme was used in all subsequent reactions.

3.2.3 Estimation of efficiency of HAV RT-PCR

In order to determine the efficiency of the reverse transcription and PCR, a known concentration of RNA encoding the VP1:2A junction was titrated using the optimised method. To accurately quantitate RNA by UV spectroscopy, a concentration greater than 20 μg ml^{-1} is required. Initially, extraction of RNA from high titre preparations of tissue cultures infected with HAV HM175A.2 was attempted. RNA was extracted by the

proteinase K / SDS method. However, insufficient yields were obtained for accurate quantitation. Therefore, in order to produce the large titre of RNA for quantitation by UV spectroscopy, a PCR derived 1.5 Kb area of the HAV genome including the VP1:2A junction was cloned into a pGEM vector (Promega, Figure 6.11) and transcribed using SP6 RNA polymerase.

Figure 3.11 pGem-T vector map

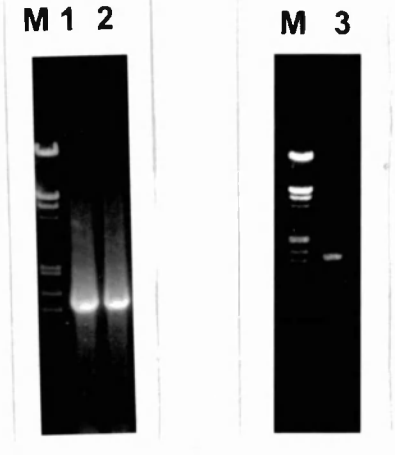


3.2.3.1 Purification of PCR products

RNA from a tissue culture extract of HAV HM175A.2 was extracted, reverse transcribed and amplified using primers A5 (forward, nucleotides 1918-1937 5' -AGC AAG CAA CTA CTG CTC C-3') and A8a (reverse, as described) exactly as previously described. The resulting 1.5 kb product covers the VP1 / 2A junction of the HAV genome. After electrophoresis on ethidium bromide stained, 1 % agarose gel amplification products

were purified using a freeze-squeeze method optimised by Tautz and Renz (1983). The appropriate band of DNA was cut from the gel under UV illumination, and placed in a 1.5 ml microtube. The tube was then filled with 0.3 M sodium acetate (pH 7.0), 1mM EDTA and gently agitated in the dark for 45 min. The gel slice was then placed on a spin-X column (Costar, Cambridge , MA) and immersed in a solid carbon dioxide / ethanol bath until the gel was completely frozen (~30 min). The column was then immediately centrifuged at 13000 xg for 10 min. The recovered DNA was precipitated by the addition of 2.5 volumes of ethanol and incubation in a solid carbon dioxide / ethanol bath for 30 min. Precipitated DNA was pelleted by centrifugation at 13000 x g for 20 min and washed twice in 1 ml 70 % ethanol. The DNA pellet was vacuum dried, resuspended in TE buffer and analysis by agarose gel electrophoresis (Figure 3.12).

Figure 3.12 Purification of amplification products



Samples

M	molecular weight markers 1
1+2	A5/A8a PCR product
3	Purified PCR product

3.2.3.2 Ligation

The freeze squeeze purified PCR product was cloned into the pGEM-T vector (Promega) according to manufacturers instructions. Vector pGEM-T is Promega's pGEM-5Zf(+) vector, EcoR V cut with 3' terminal dTTP added to each end. Since several thermostable DNA polymerases generate 5' dATP overhangs, products of amplification can ligated

into this vector directly after purification. Freeze squeeze purified DNA (30 ng) was ligated to 50 ng of vector in a 10 µl reaction mix containing 1 x ligase buffer (30 mM Tris HCl pH 7.8, 10 mM magnesium chloride, 10 mM DTT, 1 mM ATP) and 3 Weiss units T4 DNA ligase (supplied with the vector). The ligation mix was incubated at 4 °C overnight prior to transformation of E.*coli* DH5α cells.

3.2.3.3 Production of Competent cells

E. *coli* DH5α cells were made competent for transformation by the method of Hanahan (1983) using aseptic techniques throughout. A 5 ml overnight culture of E. *coli* DH5α cells in SOB was grown at 37 °C, shaking at 275 rpm. A sterile 1 l flask was rinsed with 100 ml SOB. A further 100 ml SOB was subsequently added to the flask and inoculated with 0.2 ml of the overnight culture. The flask was incubated as above until the absorbance of the cell suspension at 550 nm was approximately 0.5 (~ 3 hr incubation). All subsequent stages were done at 0-4 °C. The cell suspension was then equally divided between 2 pre-chilled 50 ml polypropylene centrifuge tubes and centrifuged at 2.5 krpm, 4 °C for 15 min. The supernatant was removed and ice cold FSB was added to 1 / 3 of the original volume. The cells were resuspended gently by carefully pipetting up and down a sterile Pasteur pipette. Cells were pooled, centrifuged at 2.5 krpm, 4 °C for 15 min and the pellet resuspended in 1 / 12.5 the original volume FSB. Dimethylsulphoxide (DMSO) was added to 3.3 %, the cells swirled gently and incubated on ice for 10 min. A further equal volume of DMSO was added and after swirling, the cells were incubated as above for 15 min. Cells were aliquoted in 200 µl volumes frozen in a solid carbon dioxide / ethanol bath and stored at -70 °C.

The efficiency of the competent cells was tested using purified plasmid pUC19 DNA (Beohringer Manheim). Transformation with 2 pg of the plasmid produced 226 blue colonies giving a transformation efficiency of 1.13×10^8 colonies per µg of plasmid.

3.2.3.4 Transformation of competent E.*coli* DH5α cells

For each transformation, a 200 µl aliquot of competent DH5α cells in a 2 ml microtube was taken from -70 °C , thawed on ice. All of the ligation mix was added whilst gently swirling the cells and the mixture incubated on ice for 30 min. Cells were then heat shocked by incubation at 42 °C for 1.5 min and returning to ice. SOC was then added to give a final volume of 1ml and after swirling, tubes were incubated at 37 °C for 1 hr. Cells were dispersed by shaking every 15 min during this incubation. All of the cells were then spread onto AIX plates which were allowed to absorb the fluid for 30 min prior to inversion and incubation overnight at 37 °C.

3.2.3.5 Small scale purification of plasmid from transformed E.*coli* DH5α cells

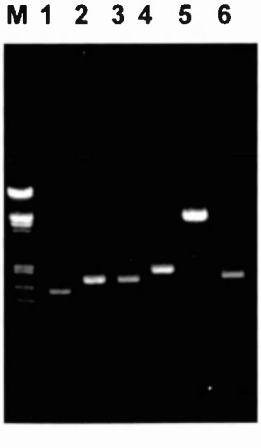
Plasmid was purified using a QIAprep Spin Miniprep Kit (QIAGEN Ltd, Crawley, UK) according to manufacturers instructions. Five ml of Luria Bertani broth containing 100 µg ml^{-1} ampicillin were inoculated with single bacterial colonies from transformed E.*coli* DH5α cells and grown overnight at 37 °C. The cultures were pelleted by centrifugation at 2600 x *g*, 4 °C for 10 min. The supernatant was removed, the cells resuspended in 250 µl buffer P1 and transferred to a 1.5 ml microtube. An equal volume of buffer P2 was then added and mixed by gentle inversion. Chilled N3 buffer (350 µl) was added and the tube again mixed by inversion. The mixture was incubated on ice for 10 min prior to centrifugation at 13400 x *g* for 10 min. The supernatant was applied to a QIAprep column which was placed in a 2 ml collection tube. The column was centrifuged at 13400 x *g* for 1 minute and the flow through discarded. The column was washed with 750 µl PE buffer and centrifuged as before. Again the collection tube was drained and the column centrifuged a further minute at 13400 x *g*. Fifty µl 10 mM Tris HCl pH 8.0 was applied to the column which was incubated for 1 minute at ambient

temperature before placing in a clean 1.5 ml microtube and centrifuging as above. The eluted plasmid was stored at -20 °C.

Each of the plasmid preparations were analysed by restriction digestion in order to determine whether the HAV PCR product had been inserted. In 10 µl reaction mixes, 1 µl of the eluted plasmid was incubated with 5 units of enzyme Bam HI at 37 °C for 1 hr. Half of each restriction digestion mix was then run on an ethidium bromide stained, 1 % agarose gel. The pGEM plasmid does not contain a restriction site for Bam HI whereas the HAV PCR product does, therefore, only plasmids with the insert will be restricted. Of 6 colonies picked, only 1 contained the pGEM plasmid with desired insert (Figure 3.13, lane 5). The size of this plasmid, designated pGEM-HAV5, was found to be approximately 4.5 kbp by comparison with the molecular weight markers as expected (Vector - 3 kbp, insert - 1.5 kbp). Uncut plasmids appear to run with a lower molecular weight due to the super coiled nature of these nucleic acids. By comparison of the intensity of the ethidium bromide stained band with those of the markers after electrophoresis, the concentration of pGEM-HAV5 was determined to be 40 µg ml^{-1}.

Figure 3.13 Restriction enzyme digestion products from small scale plasmid preparations

All small scale plasmid preparations were digested with restriction enzyme Bam HI.



Samples

M	Molecular weight markers 1
1	pGEM-HAV clone 1
2	pGEM-HAV clone 2
3	pGEM-HAV clone 3
4	pGEM-HAV clone 4
5	pGEM-HAV clone 5
6	pGEM-HAV clone 6

3.2.3.6 Large scale preparation of pGEM-HAV5

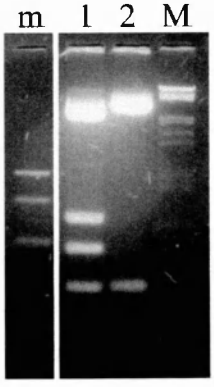
A large scale preparation of pGEM-HAV5 was purified using a QIAfilter Plasmid Midi Kit (QIAGEN Ltd, Crawley, UK) according to manufacturers instructions. E. *coli* DH5α cells, transformed with plasmid pGEM-HAV5 were harvested from a 30 ml overnight culture by centrifugation at 4 °C for 15 min at 6000 x *g*. The drained cell pellet was resuspended in 4 ml of buffer (P1) containing RNase A, mixed with 4 ml of lysis buffer (P2) containing sodium hydroxide and SDS and incubated at room temperature for 5 min. The lysate was neutralised by the addition of ice cold 4 ml buffer P3, containing acidic potassium acetate which causes precipitation of proteins, chromosomal DNA, cellular debris, and SDS and incubation on ice for 15 min. The precipitate was pelleted by centrifugation at 4 °C for 30 min at 30000 x *g*. The clear supernatant was applied to a pre equilibrated QIAGEN-tip 100 column which was allowed to empty by gravity flow. After washing the column 3 times with 10 ml of buffer QC, the plasmid DNA was eluted by the addition of 5 ml of buffer QF. The eluted DNA was precipitated by the addition of 0.7 volumes of isopropanol and centrifuged immediately at 4 °C for 30 min at 15000 x *g*. The pellet was washed with 5 ml ice cold 70 % ethanol, air dried for 5 min and resuspended in 100µl TE buffer.

3.2.3.7 Restriction digestion analysis

In order to determine the orientation of the cloned HAV DNA insert in pGEM-HAV5, two restriction digestions were performed. In the first reaction mix, the plasmid was cut with enzymes Bam HI, Hind III and Sac I and in the second mix with Bam HI, Hind III and Sph 1. In each reaction 20 µl mix, 2 µl of the eluted plasmid was digested by incubation for 1 hr at 37 °C in 1 x 'One-phor-all' restriction enzyme buffer (Pharmacia), containing 2 units of each of the appropriate restriction enzymes. Five µl from each

reaction mix was then electrophoresed on a ethidium bromide stained 1 % agarose gel (Figures 3.14 and 3.15).

Figure 3.14 Restriction enzyme digestion of products from large scale preparation of plasmid pGEM-HAV5



Samples

m molecular weight markers 2
1 Sac I, Hind III and Bam HI cut pGEM-HAV5
2 Sph I, Hind III and Bam HI cut pGEM-HAV5
M Molecular weight markers 1

Figure 3.15. Estimation of the size of restriction digest products

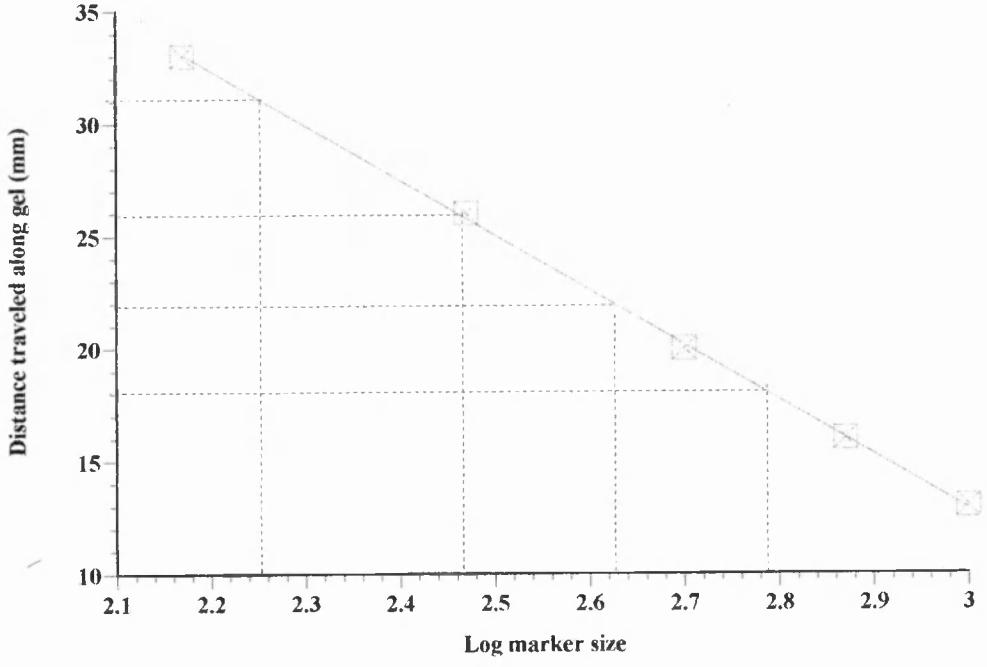


Table 3.6. Restriction analysis of pGEM HAV-5

Reaction Mix	Band	Distance travelled (mm)	Band size (b)	Expected	
				SP6	T7
A: Sac I, Hind III and Bam H I	1	26	290	190	300
	2	22	425	300	430
	3	18	610	620	620
B: Sph I, Hind III and Bam H I	1	31	180	300	170
	2	26	290	1200	300

The results of restriction digestion analysis revealed that the HAV insert in pGEM-HAV5 was positioned such that transcription by T7 RNA polymerase resulted in the formation of positive sense transcripts.

3.2.3.8 In vitro transcription

Plasmid pGEM-HAV5 contains a T7 RNA polymerase promotor region adjacent to the multiple cloning site, therefore the HAV insert could be transcribed to RNA using T7 RNA polymerase. The plasmid also contains a SP6 RNA polymerase promotor, however, the cDNA was orientated in such a way as to produce negative strand RNA when transcribed by SP6 RNA polymerase and positive strand RNA when T7 RNA polymerase is used, making the latter polymerase the enzyme of choice.

In order to create RNA transcripts similar in size to that of the insert, 50 μg of the plasmid was first digested with 10 units of restriction enzyme Mlu-I in a 50 μl reaction mix containing 50 mM Tris HCl, 10 mM magnesium chloride, 100 mM sodium chloride and 1 mM DTT, pH 7.5. After incubation at 37 °C for 1 hr , 2 μl of the reaction mix was analysed by electrophoresis on an ethidium bromide stained 1% agarose gel to check for complete digestion of the plasmid. The volume of the reaction mix was increased to 200 μl with TE buffer and the restriction enzyme removed by extraction with an equal volume of phenol / chloroform followed by a chloroform extraction. The linearised plasmid was precipitated by incubation with 0.1 vol 3 M sodium acetate and 2.5 volumes ethanol on ice for 30 min and pelleted by centrifugation at 15000 x *g* for 20 min. The pellet was washed with 5 ml ice cold 70 % ethanol, air dried for 5 min and resuspended in 20 μl water.

The cut plasmid was transcribed in *vitro* using a RiboMAX transcription kit (Promega). In a total volume of 20 μl containing 80 mM HEPES buffer pH 7.5, 32 mM magnesium

chloride, 40 mM DTT, 2 mM spermine and 7.5 mM of each of ATP, CTP, GTP and UTP, 10 µl of the linearised plasmid was transcribed using 30 units of T7 RNA polymerase. Ribonuclease activity was inhibited by the addition of 100 U RNAsin . The transcription reaction mix was incubated at 37 °C for 2 hr after which the DNA template was destroyed by incubation with 2 U RQI RNase free DNase (Promega) for a further 30 min. The volume of the reaction mix was increased to 500 µl by the addition of DEPC treated water and the enzymes removed by extraction with an equal volume of phenol / chloroform followed by chloroform extraction. Unincorporated nucleotides and degraded DNA template were removed by ethanol precipitation. A 0.5 volume of 7.5 M ammonium acetate and 2 volumes of ethanol were added to the RNA sample. The sample was mixed, placed at -20 °C for 30 min and centrifuged at 13400 x *g* for 5 min in a microcentrifuge. The pellet was washed twice with 1 ml 70 % ethanol and resuspended in 100 µl of DEPC treated water. A 1:10 dilution of the RNA was heat denatured in a boiling water bath for 5 min immediately prior to analysis on an ethidium bromide stained 1 % agarose gel (Figure 3.16). The RNA obtained was the expected size and the DNA template was not visible.

Figure 3.16 ***In vitro*** **transcribed HAV RNA**

Samples

M RNA molecular weight markers

1 *In vitro* transcribed RNA

A 1:25 dilution of the transcribed RNA was made in nuclease free water and the absorbance at 260 nm and 280 nm measured using a spectrophotometer (Kodak) in order to determine its concentration and purity.

$O.D._{260}$ of transcribed RNA (1:25) = 0.784

1 A_{260} unit = 40 $\mu g\ ml^{-1}$

$\therefore$ Concentration of transcribed RNA = 0.784 x 40 x 25 (dilution factor)

$= \underline{784\ \mu g\ ml^{-1}}$ (0.784 μg / μl)

The ratio of absorbance at 260 nm and 280 nm gives an estimation of the purity of nucleic acid with regard to protein or phenol contamination. An A_{260} / A_{280} ratio of approximately 2.0 indicates that the sample is not contaminated with any protein or phenol.

$O.D._{280}$ of transcribed RNA = 0.437

A_{260} / A_{280} = 1.8

3.2.3.9 Calculation of molecular concentration of RNA

In order to determine the concentration of transcribed RNA in terms of molecules / μl, the molecular weight of the molecules was estimated. The composition of the RNA was determined by analysis of the published sequences of wild type HAV HM175 and the pGEM vector and used in the following equation.

$$Mw = (A_n \times 347.2) + (U_n \times 324.2) + (C_n \times 307.2) + (G_n \times 363.2) + 60^a$$

[a] The addition of 60 at the end of the molecular weight calculation corresponds to the phosphate group at the 5' end of the RNA molecule.

Table 3.7 Composition of transcribed RNA

Nucleotide	HAV Insert	Transcribed region of pGEM vector	Total
A	433	17	450
C	263	33	296
G	329	34	363
U	451	15	466

Mw of transcribed RNA $= 5.3 \times 10^5$

$\therefore 5.3 \times 10^5$ g RNA $= 6.023 \times 10^{23}$ molecules (Avagadro's constant)

Concentration of transcribed RNA $= 0.784$ μg / μl

$= \underline{8.9 \times 10^{11} \text{ molecules / μl}}$

3.2.3.10 RT- PCR quantitation

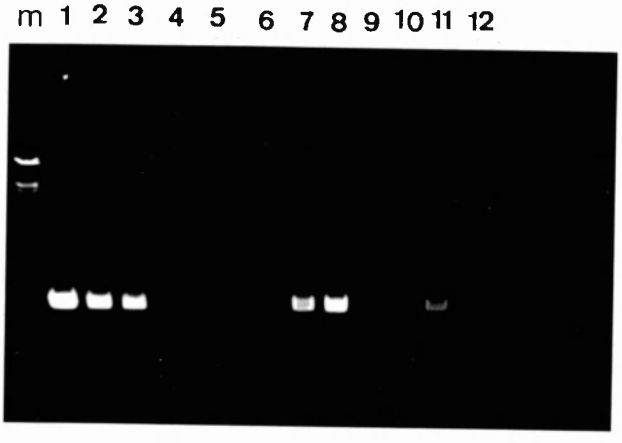
Ten fold dilutions of the RNA were prepared in nuclease free water containing 2 μg ml^{-1} carrier RNA . Of each dilution from $1:10^8$ to $1:10^{12}$, 5 μl was reverse transcribed and amplified by PCR as described in chapter 2. Analysis by agarose gel electrophoresis showed that the RNA could be detected by RT-PCR up to the $1:10^{10}$ dilution. From cDNA prepared using the $1:10^{10}$ dilution, 0.5 log dilutions were prepared in the above diluent and five replicates of dilutions from $1:10^{10.5}$ to $1:10^{12.5}$ were amplified as previously described and the results are shown in Table 3.8.

Table 3.8. Assay of transcribed RNA

Dilution	**Number of replicates positive / 5**		
	1	**2**	**3**
$1:10^{10.5}$	5	5	5
$1:10^{11}$	2	2	3
$1:10^{11.5}$	1	2	1
$1:10^{12}$	0	0	0
$1:10^{12.5}$	0	1	0
Estimated titre	10.89	11.13	10.96

Mean RT-PCR titre = 9.77×10^{10} genomes /sample

Figure 3.17 RT-PCR of ten fold dilutions of *in vitro* transcribed RNA



Samples

M molecular weight markers 1

Reverse transcribed RNA
1 $1:10^{8}$
2 $1:10^{9}$
3 $1:10^{10}$
4 $1:10^{11}$
5 $1:10^{12}$
6 Negative control water

RNA (not reverse transcribed)
7 $1:10^{6}$
8 $1:10^{7}$
9 $1:10^{8}$
10 $1:10^{9}$

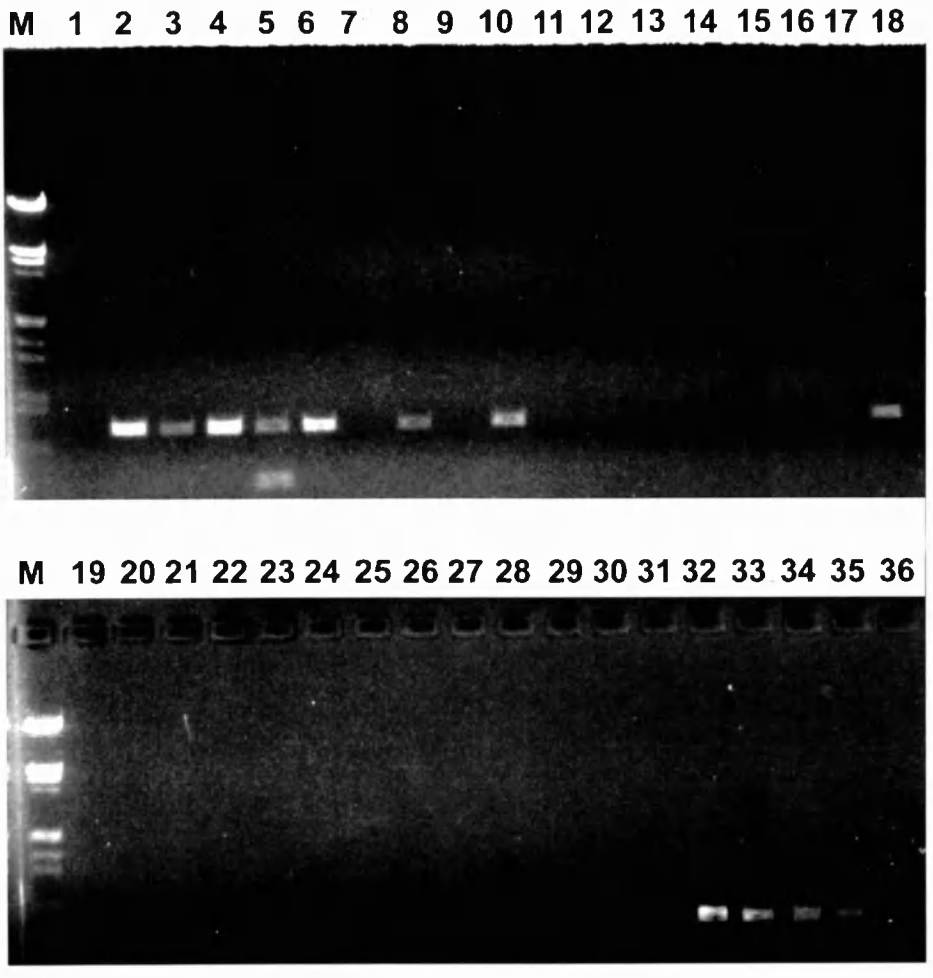
11 Positive control CS101 (1:10 dilution)
12 Negative control water

Ten fold dilutions of RNA which had not been reverse transcribed were also amplified by PCR to determine whether sufficient template DNA had been removed such that it did not interfere with the results of RNA quantitation. DNA was detected in dilutions to $1:10^{8}$ so that levels were almost 1000 fold lower than that of the RNA and therefore negligible in the calculation of the RNA titre.

3.2.3.11 Calculation of RT-PCR efficiency

Reverse transcription was done using 5 µl of sample in a 20 µl reaction mix of which 5 µl was added to the PCR mix. Therefore only 1.25 µl of the original sample was amplified.

Figure 3.18 Example PCR titration of *in vitro* transcribed RNA



Samples

M	molecular weight markers 1
1	negative control water
2-6	cDNA diluted $1:10^{10.5}$
7	negative control water
8-12	cDNA diluted $1:10^{11}$
13	negative control water
14-18	cDNA diluted $1:10^{11.5}$
19	Negative control water
20-24	cDNA diluted $1:10^{12}$
25	Negative control water
26-30	cDNA diluted $1:10^{12.5}$
31	Negative control water
32+33	Standard CS101 1:10
34+35	Standard CS101 1:100
36	Negative control water

In 1.25 µl there were 1.11×10^{12} molecules (=$1.25 \times 8.9 \times 10^{11}$)

$$\% \text{ RT-PCR efficiency} = \frac{100}{\text{Actual RNA titre}} \times \text{mean RT-PCR RNA titre}$$

$$= \frac{100}{1.11 \times 10^{12}} \times 9.77 \times 10^{10}$$

$$= \mathbf{8.8\ \%}$$

3.3 DISCUSSION

The cytopathic TCIU assay described is simple to perform, using traditional virus culture techniques and does not rely on an intricate detection system. Results achieved using this assay are comparable with those gained by plaque assay. The virus strain used (HM175A.2) has been well characterised and shown to have single hit kinetics in the cytopathic plaque assay (Anderson, 1987). The repeatability of the assay was confirmed by statistical analysis.

In the development of HAV vaccines, an adequate antibody response needs to be established. According to studies using human immune serum globulin, which is used in the prophylactic prevention of HAV, the level of serum anti HAV required for protection for several weeks may be below 20 mIU ml^{-1} (Stapleton, 1995). The ability to detect neutralising antibodies using a microtitre plate cytopathic inhibition assay opens up new possibilities for large scale seroepidemiological studies and thereby aids the rationalisation of vaccination schedules.

The use of RT-PCR quantitation has become widespread in the estimation of viral load, particularly when studying viruses which cannot easily be quantitated by infectivity based assays, such as HIV or HCV. An advantage of amplification based RNA

quantitation is the high sensitivity of RT-PCR compared with alternative techniques such as Northern blotting, RNA dot blot and RNase protection assay which require between 10^5 and 10^7 copies of the target molecule (Sambrook *et al.*, 1989; Farrell, 1993). Using an RT-PCR based method, Becker-Andre and Hahlbrock (1989) were able to reproducibly quantitate as little as 100 copies of target RNA.

Several different approaches to quantitative PCR have been reported and are either based on limiting dilution analysis or comparison of the quantity of amplicons produced compared to a standard curve of DNA or RNA which has also been quantitated by an alternative technique. For the latter approach, there is also a variety of techniques used in the measurement of amplification products including the use of radiolabelled or fluorescently labelled primers combined with analysis of electrophoresed products (Ferré, 1992;1994; Cottrez *et al.*, 1994), chemiluminescent analysis, involving the use of biotinylated primers to allow capture of the amplicons in a streptavidin coated microtitre plate and hybridisation of specific, alkaline phosphatase labelled oligonucleotide probes (Suzuki *et al.*, 1992; Whitby and Garson, 1995). Since the equipment required for accurate quantitation of amplicons was not available, the limiting dilution approach to RT-PCR was adopted.

Concerns have been raised as to the accuracy of quantitative amplification of RNA due to variability in the efficiency from 5 -90 % of the reverse transcription step (Noonan *et al.*, 1990; Henrard *et al.*, 1992; Simmonds *et al.*, 1990). In order to overcome this problem, a HAV standard was produced which was included in every RNA extraction, reverse transcription and PCR reaction so that the performance of each assay could be monitored. The performance of this standard over 20 assays suggests that there is little between assay variation in RT-PCR efficiency. The efficiency of the RT-PCR method

was also determined using *in vitro* transcribed HAV RNA which had been quantitated by UV spectroscopy. The results of 3 separate quantitative RT-PCR assays of the RNA showed that the efficiency of this method varied from 7-10 % with a mean of 8.8 %. The mean value of the efficiency of RT-PCR was taken in to account in subsequent estimations of HAV RNA copy numbers.

Chapter 4

INACTIVATION AND ELIMINATION OF HAV IN FACTOR VIII

4.1 INTRODUCTION

The transmission of HAV by blood transfusion is rare and until recently there was no evidence of transmission of the virus via plasma products (Gerety, 1982). However, outbreaks of hepatitis A since 1989 amongst haemophiliacs receiving solvent detergent treated clotting factor concentrates have caused concern to manufacturers of blood products and control agencies. Possible transmission of HAV by clotting factor concentrates was first reported in Italy by Mannucci (1992). Subsequently the use of solvent/detergent treated clotting factors was implicated in clusters of hepatitis A occurring in haemophiliac populations of Germany (Gerritzen *at al.*, 1992), Belgium (Peerlink and Vermylen, 1993), Ireland (Temperely *et al.*, 1993) and the United States (MMWR,1996). Solvent/detergent treatments are used to destroy contaminating viruses during the manufacturing process by destroying the lipid viral envelope. Since HAV does not possess a lipid envelope, it is resistant to solvent detergent inactivation as is Parvovirus B19, another non-enveloped virus of concern in blood virology. Alternative or additional virus inactivation or elimination approaches are now being assessed.

Wild type strains of HAV grow poorly in tissue culture and therefore infectivity titres cannot be measured. Tissue culture adapted strains are available which can be used in viral inactivation or elimination experiments, however, the widely used assays take up to 2 weeks to complete and often are complex involving the used of labelled antibody detection systems. The polymerase chain reaction (PCR) offers a more rapid viral detection method for such investigations and can be used to detect wild type HAV, however, the results are difficult to interpret in terms of potential infectivity.

The European Medicine Evaluation Agency (EMEA) have recently adopted a plan for the implementation of plasma pool testing for hepatitis C RNA by gene amplification

technology prior to use in the manufacture of blood products. Testing for other viruses such as HIV, HBV and parvovirus B19 by nucleic acid amplification methods has also been proposed by the EMEA and is likely to be adopted in the future. The recent outbreaks of HAV amongst recipients of blood products has also brought about plans to test plasma pools for HAV RNA by RT-PCR as wild type HAV cannot be easily measured by infectivity assay.

The terminal dry heat inactivation of HAV has been assessed in spiked factor VIII by RT-PCR. The results of nucleic acid quantitation have also been compared to infectivity data in order to determine the value of gene amplification technology to the area of validation of viral inactivation procedures and screening of plasma pools. The fate of HAV in spiked factor VIII during the affinity purification technique used in the production of Replanate (BPL, Elstree,UK) was also studied by HAV RT-PCR of material at each stage of the process.

Picornaviruses are typically highly susceptible to heat inactivation (Wallis and Melnick, 1962). The nature of the effect of heat on poliovirus and rhinovirus in suspension has been demonstrated by electron microscopy (McGregor and Mayor, 1970). At 45 °C, the poliovirus capsid becomes unstable with holes appearing in the structure after only 30-60 seconds. Heating to 50 °C for 10 minutes resulted in the appearance of empty capsids and naked RNA. Rhinovirus 14 was found to be a little more stable than polioviruses. The majority of rhinovirus particles remained intact after heating to 45 °C for 1 minute. However, at 50 °C, release of RNA from the rhinovirus capsids occurred quickly. Siegl and co-workers (1984) showed that HAV has a higher thermostability than poliovirus. The temperature at which 50 % of poliovirus particles were inactivated after a 10 minute incubation was 43 °C whereas that of HAV was 61 °C. The addition of 1 M $MgCl_2$

elevated the temperature required for 50 % inactivation in 10 min to 61 °C for poliovirus and 81 °C for HAV.

The themostability of viruses may also be increased by lyophilisation. FMDV in blood droplets was found to be susceptible to thermal inactivation at 80 °C for 30 seconds. However, heating to 86 °C for 8 min was required for complete inactivation of the virus in lyophilised blood powder (Forbes and Cottral, 1969). Since HAV is more stable to high temperatures than other picornaviruses, in this study, temperatures between 80 °C and 100 °C were selected for dry heat inactivation studies.

4.2 TERMINAL DRY HEAT INACTIVATION STUDY 1

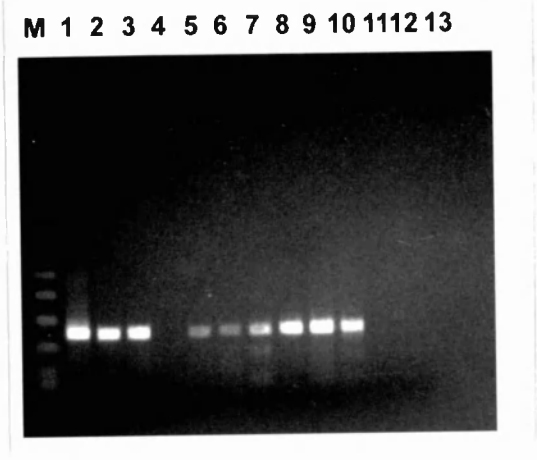
In this study, the dry heat inactivation of HAV in spiked factor VIII, as detected by a radioimmunofocus assay (RIFA), was compared with the loss of HAV RNA as determined by quantitative PCR assay. As this study was initiated prior to the development of the cytopathic microtitre plate assay previously described, the strain of HAV used was suitable for RIFA and could not subsequently be used in the cytopathic assay. Samples used in this experiment were taken from a previous investigation of the effect of dry heat treatment on HAV spiked factor VIII (Hart *et al.*, 1994a). The temperatures and time points chosen were those most likely to ensure the inactivation of HAV whilst maintaining the activity of the factor VIII.

High purity solvent detergent treated Factor VIII prepared by the Scottish National Blood Transfusion Service (SNBTS, Edinburgh, UK) from serum cryoprecipitate (Burnouf *et al.*, 1991, Hart *et al.*, 1994a) was spiked with tissue culture adapted HAV strain HM175 SL18F (Kindly provided by S. Lemon). After freeze drying in 1 ml aliquots, samples were heated at either 80 °C or 90 °C. Samples were taken at different time intervals (80 °C - 24 hr, 48 hr, 72 hr; 90 °C - 2 hr, 6 hr, 10 hr) and stored at 4 °C. The spiking and heat

inactivation steps were performed by Dr H. Hart at the Protein Fractionation Centre SNBTS. Prior to analysis, samples were reconstituted in sterile distilled H_2O and subsequently stored at -70 °C. RIFA was performed by Ms A. John (NIBSC) the results of which have previously been published (Hart *et al.*, 1994b). Samples were diluted at a minimum of 1:10 and 100 µl of inoculum was used in each dish therefore the minimum detectable titre was 10^2 RFU ml^{-1}. A 250 µl volume of each sample extracted by the proteinase K/SDS method as detailed in Chapter 2. All of the extracted RNA was reverse transcribed and a 5 µl the cDNA was used in PCR reactions as previously described, using primers HA1F and HA1R (appendix B). The limit of detection of the assay based on the quantity of material used and the efficiency of the assay is 2.3 log genomes ml^{-1}.

The results of this study are summarised in Table 4.1. Titres of virus in the stock HM175A.2 preparation and spiked factor VIII quantified by PCR titrations were similar to those obtained by RIFA. HAV infectivity in spiked factor VIII samples was reduced by 100 fold upon freeze drying. The titre of viral RNA in freeze dried factor VIII decreased more slowly than the drop in infectivity. However, after 24 hr at room temperature post freeze drying the RNA and infectivity titres were more comparable. Heating freeze dried samples to 80 °C for 24 hr or 90 °C for only 2 hr was sufficient to completely eliminate HAV tissue culture infectivity as defined by RIFA. Although levels of viral RNA were reduced by 1 log after 48 hr at 80 °C (4.1 log genomes ml^{-1}) and by 1.5 log after 6 hr at 90 °C (3.6 log genomes ml^{-1}) the HAV genome was still detected in the samples after each of the heat treatments (Figure 4.1). A 3.6 log_{10} genomes decrease in HAV RNA titre was achieved by combined freeze drying and heating samples to 80 °C for 72 hr whereas when lyophilised samples were heated to 90 °C for only 10 hr the titre was reduced by 4.1 log_{10} genomes. Figure 4.2 shows an example quantitative RT-PCR assay of HAV RNA in spiked factor VIII.

Figure 4.1 Terminal dry heat inactivation of HAV in spiked factor VIII (SNBTS)



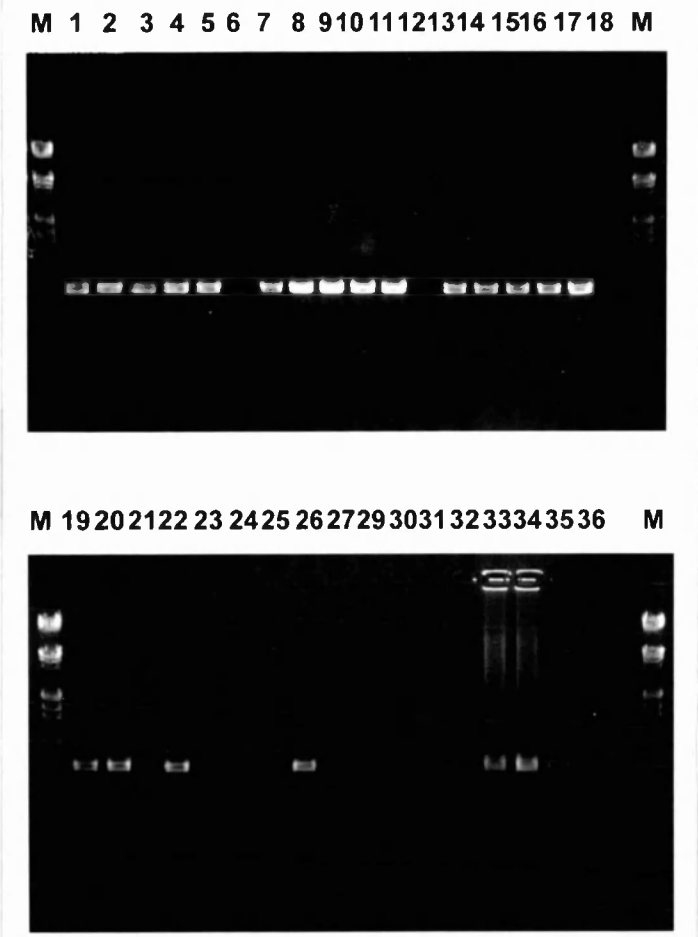
Samples

M	molecular weight markers 2	Thermal inactivation of factor VIII	
1	HAV spiked factor VIII	5	80 °C - 24 hr
2	Post freeze drying	6	80 °C - 48 hr
3	24 hr post freeze drying	7	80 °C - 72 hr
		8	90 °C - 2 hr
4	Negative control water	9	90 °C - 6 hr
		10	90 °C - 10 hr
		11	Negative control water

Table 4.1. Terminal dry heat treatment of HAV spiked factor VIII

Sample		Infectious HAV log_{10} RFU ml^{-1}	HAV RNA log_{10} genomes ml^{-1}
Stock HAV HM175A.2		6.3-7.3	7.5
HAV spiked factor VIII		6.7	7.3
Post freeze drying		4.4	7.2
24hr post freeze drying		4.3	5.1
80 °C	24hr	<2.0	5.1
	48hr	<2.0	4.1
	72hr	<2.0	3.7
90 °C	2hr	<2.0	6.1
	6hr	<2.0	3.6
	10hr	<2.0	3.2

Figure 4.2 Example quantitative PCR titration of HAV spiked factor VIII



Samples (dilutions of spiked factor VIII)

M	molecular weight markers		
1-5	cDNA diluted $1:10^{3.5}$	19-23	cDNA diluted $1:10^{5}$
6	negative control water	24	Negative control water
7-11	cDNA diluted $1:10^{4}$	25-29	cDNA diluted $1:10^{5.5}$
12	negative control water	30+32	Negative control water
13-17	cDNA diluted $1:10^{4.5}$	33+34	Standard CS101 1:10
18	negative control water	35+36	Standard CS101 1:100

Calculation of HAV RNA titre

Result of GLIM analysis = 10^5 genomes / sample

log RNA titre ml^{-1} = $\log (10^5 \times (100/8.8)^a \times (1000/62.5)^b)$

= 7.3 log genomes ml^{-1}

[a] Replication efficiency factor
[b] Volume factor (Proteinase K / SDS extraction)

4.3 TERMINAL DRY HEAT INACTIVATION STUDY 2

A second study of terminal dry heat inactivation was performed using factor VIII samples prepared at Bio Products Laboratories (BPL, Elstree, London). The factor VIII (Replenate) was prepared from cryosupernatant and purified by monoclonal antibody affinity purification and has been shown to be more stable at high temperatures than the factor VIII prepared at SNBTS (Roberts, 1995). On heating the Replanate factor VIII to 72 °C for up to 72 hr, less than 10% of the clotting activity was lost compared to a 33.8 % loss seen with the SNBTS preparation (Hart *et al.*, 1994b). This study was performed in order to exammine whether determinants which increased the thermal stability of the factor VII also conferred greater viral stability. The inactivation of HAV at 80 °C at 24 hr, 48 hr and 72 hr were performed for direct comparison with the previous study. Earlier time points of 2 hr and 8 hr were also included to determine whether the length of incubation time at this temperature could be reduced whilst still inactivating HAV with regard to tissue culture infectivity. As the factor VIII sample used in this study was of greater stability than that of the previous study, the effect of heating spiked samples to 100 °C could be compared with the other inactivation temperatures used.

The factor VIII was spiked with tissue culture extracted HAV cytopathic strain HM175A.2 by making a 1:10 dilution in the sample of virus stock (10^7 TCIU ml^{-1}) to give a concentration of 10^6 TCIU ml^{-1}. The spiked factor VIII was then placed in 5 ml aliquots and freeze dried prior to heat treatment. Two vials of lyophilised factor VIII were heated at 80 °C for 2 hr, 8 hr, 24 hr, 48 hr or 72 hr at 100 °C for 2 hr, 4 hr, 8 hr, 16 hr or 24 hr. Samples were stored at 4 °C. Prior to use, samples were reconstituted in 5 ml sterile distilled H_2O and subsequently stored at -70 °C. All samples were analysed by cytopathic microtitre plate assay and quantitative RT-PCR as described in chapter 2. For the cytopathic microtitre plate assay, samples were diluted at a minimum of 1:100 as more

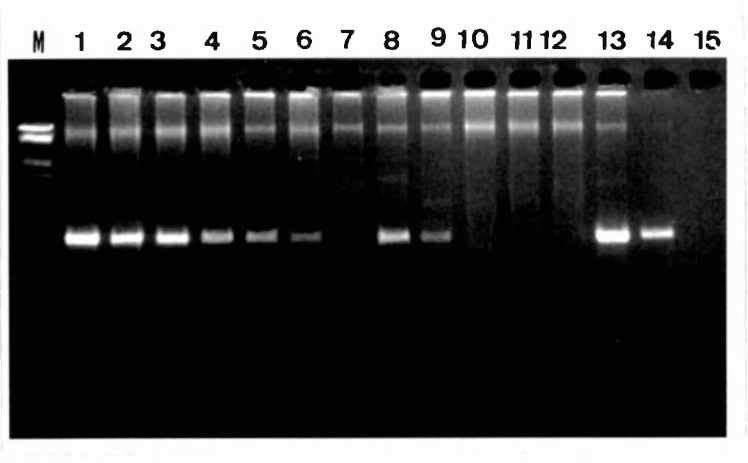
concentrated preparations resulted in lysis of BS-C-1 cells. Since 100 µl of sample was added to each well, the minimum detectable titre was 10^3 TCIU ml^{-1}. RT- PCR quantitation was performed on samples extracted by the QIAmp Viral RNA extraction kit as detailed in Chapter 2. RNA was extracted from 140 µl of each sample. A tenth of extracted RNA (5 µl out of 50 µl) was reverse transcribed and a quarter of the cDNA was PCR amplified. The detection limit of the RT-PCR protocol based on the efficiency of the system and the volume of sample used is therefore 3.5 log genomes ml^{-1}.

When HAV spiked Replenate factor VIII was dry heat treated at 80 °C for 2 hr or longer, the tissue culture infectivity was decreased from 5.3 log TCIU ml^{-1} to a level that was not detectable by the cytopathic microtitre plate assay (Table 4.2, Figure 4.3). Similarly heating lyophilised samples to 100 °C for 2 hr also abolished the infectivity. HAV RNA was however detected in all samples which had been treated at 80 °C. After 72 hr at 80 °C, a 2.9 log_{10} reduction in RNA titre was seen. Heating the spiked factor VIII to 100 °C for 8 hr was sufficient to reduce the titre of the HAV genome to a level below the limit of RT-PCR detection.

Table 4.2 Terminal dry heat inactivation of HAV in spiked Replenate factor VIII

Sample		Infectious HAV log_{10} TCIU ml^{-1}	HAV RNA log_{10} genomes ml^{-1}
HAV spiked factor VIII		6.2	6.5
Post freeze drying		5.3	6.1
80 °C	2 hr	< 3.0	5.2
	8 hr	<3.0	5.0
	24hr	< 3.0	4.2
	48hr	< 3.0	3.7
	72hr	< 3.0	3.6
100 °C	2 hr	< 3.0	4.6
	4 hr	< 3.0	3.8
	8 hr	< 3.0	<3.5
	16 hr	< 3.0	<3.5
	24 hr	< 3.0	<3.5

Figure 4.3 Terminal dry heat inactivation of HAV in spiked factor VIII (BPL)



Samples

M	molecular weight markers 1	Thermal inactivation of factor VIII			
1	HAV spiked factor VIII	3	80 °C - 2hr	8	100 °C - 2 hr
2	Post freeze drying	4	80 °C - 4hr	9	100 °C - 4 hr
		5	80 °C - 24 hr	10	100 °C - 8 hr
		6	80 °C - 48 hr	11	100 °C - 16 hr
		7	80 °C - 72 hr	12	100 °C - 24 hr
		13	Control CS101 (1:10)		
		14	Control CS101 (1:100)		
		15	Negative control water		

4.4 ANALYSIS OF THE INTEGRITY OF THE HAV RNA AFTER HEAT INACTIVATION

The RT-PCR method used in the inactivation studies quantitates only the region amplified by the set of primers used, i.e. nucleotides 2694 - 3393, spanning the VP1:2A junction of the HAV genome. No information is given as to the integrity of the complete HAV genome and it's ability to cause infection using this method. Therefore the following approach was used in order to clarify this point.

In reverse transcription, the genome is copied from the 3 prime end of the sequence to be transcribed by extension of the 5 prime end of the primer. If a specific primer is used,

only areas upstream of the primer should be transcribed and therefore available for amplification. Any break along the RNA molecule would result in the production of a truncated cDNA strand and the inability to amplify areas upstream of the break by PCR

Reverse transcription and PCR primers (Table 4.3) were designed to amplify regions toward the 3 prime and 5 prime ends of the genome as shown in Figure 4.4. The RNA extracted from tissue culture harvested HAV HM175A.2 was reverse transcribed using either primer 2, to produce cDNA corresponding to the first 2241 bases of the HAV genome, or primer 5, transcribing almost all of the genome. The reverse transcription mixes were prepared as in Chapter 2, however, instead of random primers, 10 pmole of the appropriate specific primer was added to each reaction mix. As a reverse transcription control, a reaction was also set up containing extracted HAV RNA and reverse transcriptase but no primer included in the mix. The resulting cDNA was amplified using primer sets 1and 2 or 3 and 4

Figure 4.4. Reverse transcription and PCR primers

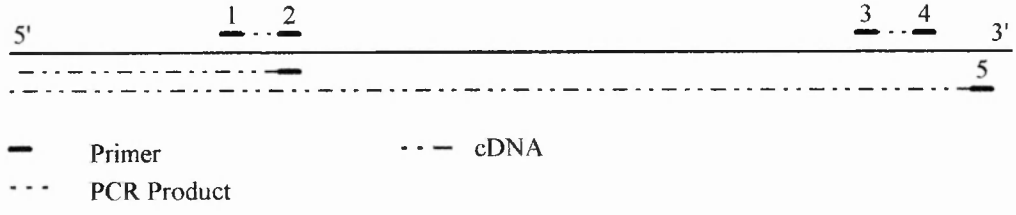
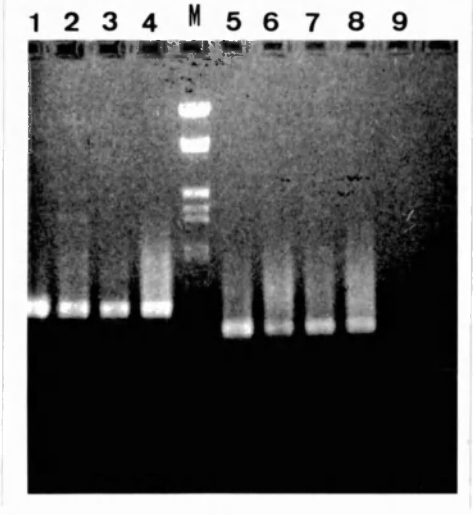


Table 4.3. Reverse transcription and PCR primer sequences

Primer	Sequence
1	1918 5' -AGC-AAG-CAA-CTA-CTG-CTC-C-3' 1936
2	2241 5'-ACT-GTT-GTT-GAA-AAA-CCT-CC-3' 2221
3	6723 5'-GAA-CGA-GGT-AGA-ATC-ATG-AG-3' 6743
4	6869 5'-TGT-ACA-AGG-AGA-CCC-AGA-G-3' 6850
5	7345 5'-TCT-ATC-ATC-TCT-TTC-TCC-AAA-CAG-GAC-3' 7319

The initial control experiments using tissue culture extracted RNA gave surprising results as shown in Figure 4.5. Positive PCR results were obtained using RNA that had been incubated with the reverse transcription mix in the absence of reverse primers 2 or 5. Sequences were also amplified from the RNA transcribed with primer 2 at the downstream position using PCR primers 3 and 4. These results may be the result of the ability of the HAV genome to "self-prime" or the presence of small complimentary nucleic acid sequences in the extracted RNA sample (Frech and Peterhans, 1994).

Figure 4.5 Non specific priming of HAV RNA during reverse transcription



Samples (HM175A.2)

M molecular weight markers 1

PCR primers 1 + 2
1 Random primed cDNA
2 RT primer 2
3 RT primer 5
4 No RT primers

PCR primers 3 + 4
5 Random primed cDNA
6 RT primer 2
7 RT primer 5
8 No RT primers
9 negative control water

In order to overcome the problem of non-specific priming, several different approaches were tried (Table 4.4). Initially, the approach used by Frech and Peterhans (1994) was attempted. Ethanol precipitated RNA extracted from tissue culture harvested HAV strain HM175A.2 by the proteinase K / SDS method described in chapter 2 was resuspended in 20 µl 6 x SSC containing 7.4 % formaldehyde and incubated at 75 °C for 5 min to denature the RNA and quenched on ice prior to spotting 1 µl of the RNA onto 8 nitrocellulose filters (0.5 cm^2, pore size 0.45 µm, Schleicher and Schuel, Dassel,

Germany). The RNA was fixed to the filters by oven drying for 3 hr at 80 °C. The immobilised RNA was reverse transcribed by the addition of the filters to 20 µl of reverse transcription mix as described in chapter 2 containing either 0.5 µg ml^{-1} random primers, 40 pmoles / reaction specific reverse primers 2 or 5, or no primers. Following reverse transcription at 42 °C for 30 min, the mixes were denatured at 80 °C for 5 min and quenched on ice prior to PCR amplification using 10 pmole / reaction of either primer set 1 and 2 or set 3 and 4.

Alternative or additional extraction methods were also examined. The QIAmp extraction method was performed as described in chapter 2. Proteinase K / SDS extracted RNA was also further purified using Microspin S400HR columns to remove small nucleic acid molecules according to the manufacturers instructions. The column resin was resuspended by vortexing, the cap loosened one fourth of a turn and the bottom closure removed. The column was placed in a 1.5 ml microtube and spun in a microcentrifuge at 735 x *g* for 1 min. The column was then transferred to a clean tube and the RNA sample, which had been resuspended in 50 µl nuclease free water, was carefully added to the top of the resin and spun as above. The purified RNA was collected in the bottom of the support tube and heat denatured as previously described prior to reverse transcription.

A commercial kit designed to extract poly adenylated RNA were also used to extract the viral RNA from other nucleic acid molecules after proteinase K / SDS extraction. The PolyATract mRNA isolation kit (Promega) was used according to manufacturers instructions. The ethanol precipitated RNA pellet was resuspended in 24 µl of ribonuclease free water and incubated at 65 °C for 10 min immediately prior to the addition of 1 µl of biotinylated oligo dTTP probe and 6 µl of SSC. The mixture was allowed to cool to room temperature for 30 min. A 50 µl volume of streptavidin coated

paramagnetic beads was washed in 150 µl 0.5 x SSC. A magnet was used to draw the beads to the bottom of the tube so that the wash buffer could be removed. After 3 washes beads were resuspended in 50 µl 0.5 x SSC and the annealing reaction mix added to the beads. The mixture was incubated at room temperature for 10 min. A magnet was then used to capture the particles at the bottom of the tube so that supernatant could be removed and the particles were washed 3 times in 0.1 x SSC (150 µl / wash). After the final wash, the particles were resuspended in 100 µl of nuclease free water and then magnetically captured. The supernatant was transferred to a clean 0.5 ml microtube and centrifuged at 13400 x *g* for 10 min to pellet any remaining particles. The resulting RNA was reverse transcribed and amplified as previously described.

Table 4.4. Prevention of non-specific priming during reverse transcription of HAV RNA

RT PRIMERS	Random		5 (3' end)		2 (5' end)		None	
PCR PRIMERS	**1+2**	**3+4**	**1+2**	**3+4**	**1+2**	**3+4**	**1+2**	**3+4**
Expected Result	+	+	+	+	+	-	-	-
RNA Extraction								
Proteinase K, SDS digestion and Phenol chloroform extraction	+	+	+	+	+	+	+	+
QIAamp viral RNA extraction kit (Qiagen)	+	+	+	+	+	+	+	+
Further RNA purification								
RNA immobilised on nitrocellulose filter	+	+	-	-	-	-	-	-
Microspin columns (Pharmacia)	+	+	+	+	+	+	+	+
Poly-A-tract mRNA isolation kit (Promega)	+	+	+	+	+	+	+	-

Table 4.4 shows that nitrocellulose membrane immobilisation was the only method to completely remove the priming of reverse transcription when no primers were added to the RT mix. However, using this method, the yield of PCR product using random primed cDNA was much lower than expected and the specific reverse primers both failed to produce sufficient cDNA to be detected by PCR. All of the other methods resulted in PCR products , even when no primers were added to the reverse transcription step.

However, no amplification was detected when RNA purified by the PolyAtract kit was transcribed without primers and amplified with primers 3 and 4.

An alternative approach was the use of an oligonucleotide poly dTTP probe to capture poly adenylated RNA only. HAV RNA is poly adenylated at the 3 prime end, therefore any HAV genomes with an intact 3 prime end could be captured and purified from other RNA fragments. Reverse transcription and PCR of the HAV RNA at the 5 prime end would show the presence of full length genomes.

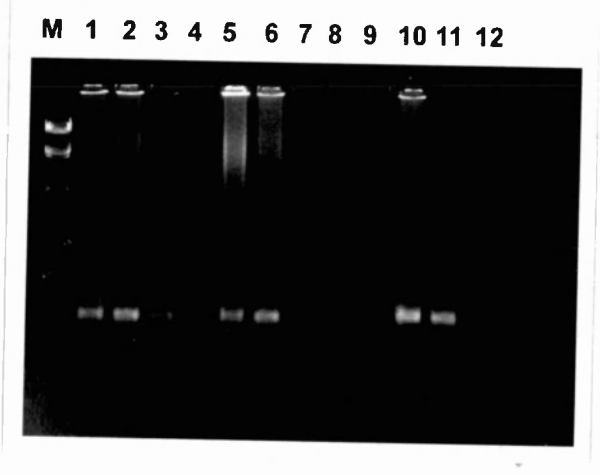
Total RNA from SNBTS factor VIII samples post freeze drying and after heating to 80 °C for 24 hr (Table 4.1) using the QIAmp HCV RNA extraction kit as previously described. The poly adenylated RNA was purified using an Oligotex mRNA kit (QIAGEN Ltd) according to the manufacturers instructions. The volume of RNA was increased to 250 µl by the addition of HPLC grade water and 250 µl of 2 x binding buffer was added followed by 15 µl of Oligotex beads (poly dTTP oligonucleotides bound to polystyrene latex particles). The mixture was incubated at 65 °C for 3 minutes to denature the RNA and then cooled to room temperature for 10 minutes allowing the hybridisation of poly adenylated RNA to the oligo dTTP. The RNA was then centrifuged for 2 min at 13400 x *g* and the supernatant carefully removed. The pelleted beads were resuspended in 400 µl of wash buffer and transferred to a spin column which was placed in a 1.5 ml microcentrifuge tube and centrifuged at 13400 x *g* for 30 seconds. Two further washes were performed by adding 400 µl of wash buffer to the spin column and centrifuging as above. The spin column was placed in a clean tube and 50 µl of HPLC grade water containing carrier RNA (0.3 µg ml^{-1}) which had been heated to 70 °C was added. the spin column was centrifuged at 13400 x *g* for 30 seconds to elute the poly adenylated RNA.

The poly adenylated RNA was reverse transcribed using random primers as previously described. Serial ten fold dilutions of each of the cDNA preparations were made to 1 : 1000 and the neat and diluted cDNA was amplified using primers HA1F and HA1R at 10 pmole per reaction in the PCR mix previously described.

PCR primers (5 prime non-coding region)

	57	77
HA1F	5' -GAC-TTG-ATA-CCT-CAC-CGC-CGT- 3'	
	312	292
HA1R	5' -AGA-CTC-CTA-CAG-CTC-CAT-GCT- 3'	

Figure 4.6 Analysis of the integrity of heat treated HAV in spiked factor VIII



Samples

M Molecular weight markers 1

Spiked SNBTS factor VIII

a) 24 hr post freeze drying
1 neat
2 1:10
3 1:100
4 1:1000

b) Heated to 80 °C for 24 hr
5 neat
6 1:10
7 1:100
8 1:1000
9 negative control water
10 CS101 1:10
11 CS101 1:100

The results show (Figure 4.6) that the 5 prime non-coding region of HAV RNA could be detected to the same dilution prior to and after heating of the spiked factor VIII sample. Both samples were positive to the 1:100 dilution which, when adjusted for sample volume and efficiency of the reverse transcription, gives an approximate titre of 10^5 genomes ml^{-1}.

4.5 ELIMINATION OF HAV BY MONOCLONAL ANTIBODY AFFINITY CHROMATOGRAPHY OF FACTOR VIII

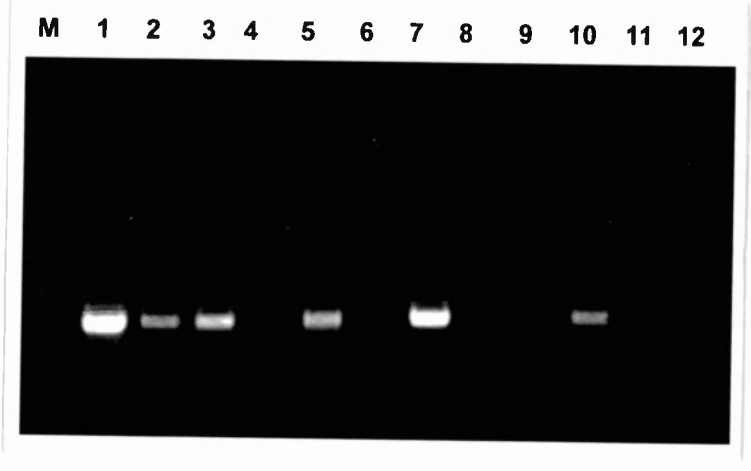
In the production of Replenate, a high purity preparation of factor VIII produced by BPL (Elstree, UK), monoclonal antibody affinity chromatography is performed on the supernatant taken after cold precipitation of cryoprecipitated plasma. In this study, the elimination of virus from HAV spiked supernatant during this process of factor VIII purification was examined by RT-PCR.

The cold precipitation supernatant was spiked with HAV strain HAV HM175/18f (stock concentration ~ 10^6 RFU ml^{-1}) at a 1:200 dilution. Samples of the spiked factor VIII were taken before loading onto the affinity purification column and 1 day after being loaded onto the column. The column flow through, wash and eluted product were also collected for analysis.

Figure 4.7 shows the results of RT-PCR analysis of each step in the process of monoclonal antibody affinity chromatography purification of factor VIII used in the manufacture of Replenate. HAV RNA was clearly detected in the load material 1 day after loading onto the column demonstrating the stability of the virus in the factor VIII preparation. A faint band of HAV RNA was detected in the material that flowed through

the column as the factor VIII was loaded, however, more of the virus appeared to be eluted in the wash buffer. Initial results of RT-PCR of the eluted factor VIII suggested that the affinity purification process had removed the virus from the preparation. When a 50 times concentrated sample of eluate (25 ml concentrated to 0.5 ml) in PBS was tested, a strong signal was obtained. After collection of eluate, sanitisation and regeneration of the column is performed so that it can be reused to purify further batches of factor VIII. The flow through of the 2 washes used in these steps were also tested for HAV RNA by PCR and found to be negative.

Figure 4.7 Elimination of HAV by monoclonal antibody affinity purification



Samples

M	molecular weight markers 2		
1	Stock HAV HM175/18f	7	50 x concentrate of eluted factor VIII
2	Spiked load material	8	Sanitisation step (NaCl)
3	Spiked load material - 24 hr after loading	9	Regeneration step (Ac)
		10	Positive control CS101 (diluted 1:10)
4	Column flow through	11	negative control water
5	Column wash		
6	Eluted factor VIII		

4.6 DISCUSSION

Several different methods which may be combined or used independently, are capable of eliminating or inactivating contaminating viruses in blood products. Events during the manufacture processes may also inadvertently lead to a reduction in virus titre such as the presence of virus specific antibodies in a proportion of the donations in a pool causing neutralisation of the contaminating virus or freeze drying of the product. Freeze drying of HAV spiked factor VIII prior to heat inactivation studies resulted in a reduction in infectious titre of 2.3 log RFU ml^{-1} and 0.9 log TCIU ml^{-1}. The transmission of HAV to haemophiliacs in recent years shows that the lyophilisation of blood products and presence of anti-HAV in the starting material is not sufficient to remove the risk of infection with this virus.

Additional steps may be added to the production of clotting factor concentrates which are designed to physically remove contaminating viruses such as affinity chromatography, ion exchange chromatography and membrane filtration. In the production of Replanate (BPL, Elstree, UK) the factor VIII is purified by affinity chromatography. The fate of HAV in spiked material was studied during this process by RT-PCR. The purification of factor VIII from the supernatant of cold precipitation of plasma cryoprecipitate reduced the level the spiked HAV. The majority of virus appeared to be eluted from the column during the washing steps. Analysis of concentrated factor VIII eluate revealed however, that not all of the HAV was removed during the purification process. Additional virus elimination or inactivation steps are therefore necessary to ensure the safety of factor VIII produced by monoclonal antibody affinity purification.

Inactivation of viruses in spiked blood products may be achieved by solvent detergent treatment (non-enveloped viruses only) and heating whilst solubilised or lyophilised.

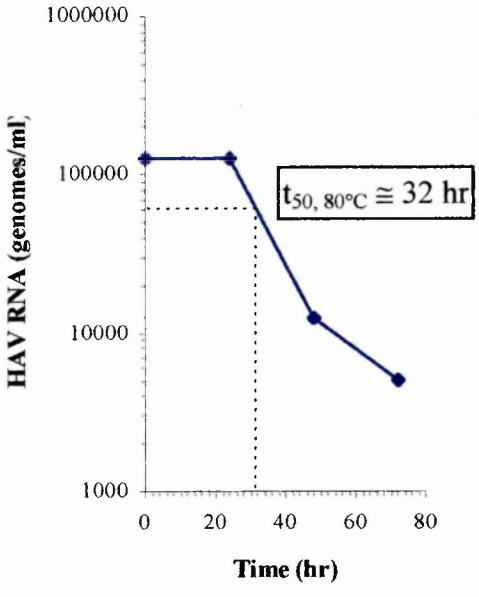
While pasteurisation of factor VIII in solution (63°C for 10 hours) results in inactivation of HAV (Schwinn *et al.*, 1994), the addition of sugars, amino acids or salts is required to prevent inactivation of the blood product. These stabilisers must be subsequently removed from the preparation resulting in an increase in the number of manipulations required in the production of the factor VIII and a potential risk of contamination after the inactivation step. Severe heating lyophilised factor VIII in the absence of stabilisers does not result in an excessive loss of product activity (Hart *et al.*, 1994b). No further processing of the factor VIII is required after dry heat inactivation. Terminal dry heat treatment of HAV spiked, freeze dried factor VIII was assessed in two studies by infectivity assays and quantitative RT-PCR. Comparison of the results obtained by detection of infectious virus and genome were used to determine the usefulness of PCR detection in blood virology.

The similarity in HAV titre determined by infectivity and PCR in untreated samples shows that the titre of HAV genome equivalents as determined by the PCR titration assay used correlates well with tissue culture infectivity. In the initial study of thermal inactivation, the delay seen in reduction of HAV RNA levels after freeze drying compared with that of infectivity may be due to slow release and subsequent ribonuclease digestion during the 24hr following disruption of virus particles upon freeze drying.

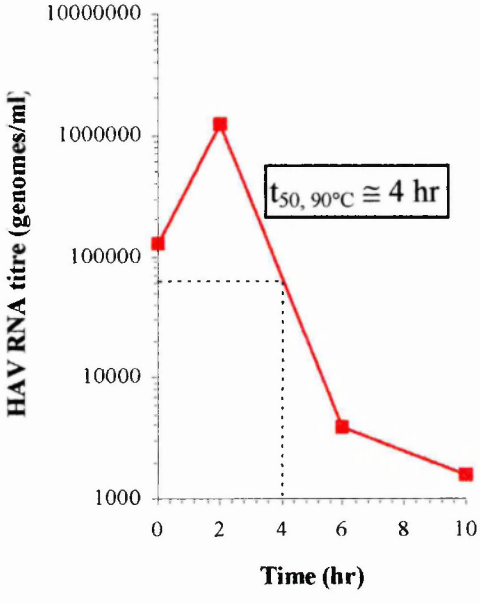
Dry heat treatment of HAV spiked SNBTS factor VIII at 80 °C for 24 hr or 90 °C for 2 hr was adequate for elimination of tissue culture infectivity as defined by RIFA. Since tissue culture infectious virus was not detected in any of the heat treated samples, it was not possible to determine the half lives of infectivity at the various temperatures used. Further inactivation studies in which samples are taken at earlier time points are required to determine the kinetics of thermal inactivation of HAV with regard to infectivity.

Figure 4.8 Half life of RNA integrity during dry heat inactivation

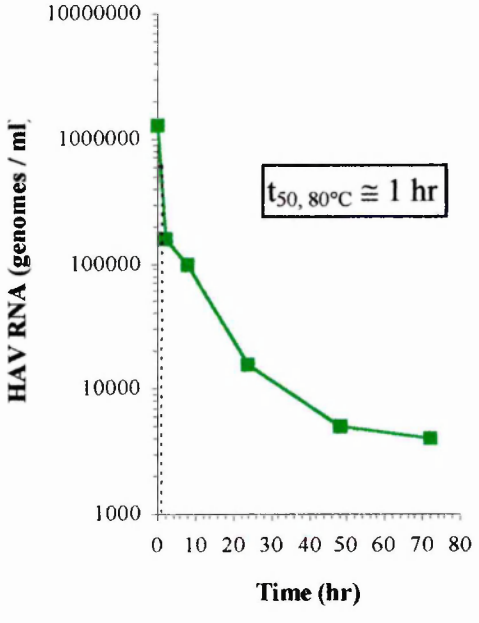
a) Study 1, 80°C



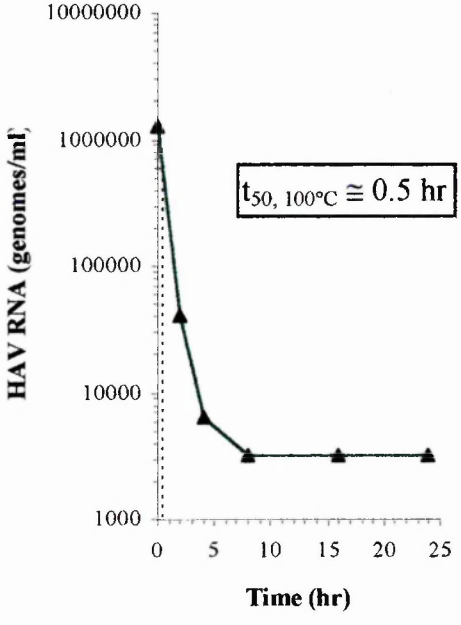
b) Study 1, 90°C



c) Study 2, 80°C



d) Study 2, 100°C



The heat treatments used in this study were not sufficient to eliminate HAV RNA as detected by RT-PCR. Figure 4.8 shows the reduction in HAV nucleic acid detection upon terminal dry heat treatment of spiked factor VIII. The calculated half lives of nucleic acid integrity are based on single experiments and therefore require confirmation in repeat studies. The kinetics of inactivation of HAV in the factor VIII from SNBTS appear to differ from those in the BPL produced factor VIII. In the initial study, there was a delay before any RNA degradation as detected by RT-PCR occurred, whereas, in the spiked factor VIII from BPL, a reduction in HAV RNA titre is seen in the first heat treated samples (2 hr at 80 °C and 100 °C). The first half life at 80 °C of the HAV genome in factor VIII from BPL is therefore much lower than that in corresponding samples from SNBTS. Since the first half lives of genome degradation observed in study 2 occurred before the initial heat treated sample was taken, in further studies, earlier time points should be sampled to more accurately determine these figures. This study does however, highlight the contribution of composition of the factor VIII preparation to kinetics of heat inactivation.

The increased thermostability of the Replenate factor VIII compared with the SNBTS product does not appear to increase the stability of spiked HAV at 80 °C. Heating the factor VIII to 100 °C for 2 hr was sufficient to eliminate tissue culture infectivity and after a further 6 hr at this temperature, viral RNA was also no longer detected. Heating at 100 °C for at least 8 hr is therefore necessary if factor VIII is required to be negative by PCR rather than infectivity. Inactivation at 80 °C for 2 hr would be sufficient based on infectivity results however, viral RNA was still detected after 72 hr at 80 °C. The loss of product activity during heat treatment may prohibit the use of temperatures as high as 100 °C in the inactivation steps of some blood products such as the SNBTS factor VIII,

therefore the importance of PCR negativity in determining the safety of blood products with regard to viral contamination must be carefully considered if blood products are to be tested at the end of manufacture.

The use of nucleic acid amplification screen for viruses in plasma pools before manufacture of blood products is favourable at this stage for several reasons. The presence of viral genome is more likely to correlate with infectivity in the starting material than after the steps involved in the manufacture of the blood product. If contaminating plasma pools are detected and removed prior to manufacture, there is less financial loss than would occur if the final product was to be destroyed. Strategies for testing of mini-pools rather than the large pools used in the manufacture of blood products would also reduce the loss of starting material and facilitate the tracing of infected donors (McOmish *et al.*, 1993).

Dry heating may cause alteration in viral capsid protein structure which prevents infection of cells. Further heat treatment being required for the RNA to be released and degraded. However, since only 699 bases of the HAV genome were amplified in the initial studies, it was unclear whether the whole RNA molecule is intact after heat treatment or only small fragments are resulting in amplification.

The initial approach used in an attempt to determine the integrity of heat treated RNA was to use specific primer to at the 3 prime non-coding region to prepare cDNA and then amplify a region towards the 5 prime end of the HAV genome. Control experiments showed that non-specific priming occurred during reverse transcription resulting in amplification of products after the reverse transcription reaction had been performed in the absence of primers. Several strategies were tried in order to remove the nucleic acids

which acted as primers for reverse transcription. The only method that completely abolished non specific priming was immobilisation of the RNA on a nitrocellulose filter. However, using this method, PCR amplification only succeeded when random primers were used in the reverse transcription. Immobilisation of the RNA on the filter may have restricted binding of the specific primers.

In an alternative approach to assess the integrity of heat treated HAV RNA, the Oligotex mRNA kit was used to capture the RNA at the 3 prime end followed by reverse transcription and PCR amplification in the 5 prime non-coding region, allowing the detection of full length RNA only. The results showed that full length RNA was present to approximately the same level in the heated factor VIII sample which no longer contained tissue culture infectious virus as in the post freeze drying sample in which RNA levels were comparable to that of infectious virus.

This study highlights the disparity between the use of infectivity assay and RT-PCR in the detection of viruses after inactivation procedures. A question which must now be addressed is whether the presence of full length HAV RNA in the blood products poses a risk to the recipients. It may be supposed that the full length RNA found in inactivated samples is encapsidated otherwise it would be quickly destroyed by ribonucleases present in the factor VIII preparation. While the heat treated virus is unable to penetrate and cause infection cells in culture, it may be possible for the intact RNA to infect cells *in vivo*. The use of nucleic acid based detection of wild type RNA in blood products may be implemented routinely in the future following the outbreaks of HAV amongst recipients of blood products. While nucleic acid amplification technology is highly sensitive, interpretation of the results from such techniques is difficult with regard to defining the risk of transmission of HAV in material which is tested positive for the viral genome. The

high demand for products derived from voluntarily donated plasma necessitates the careful management of this limited resource. Therefore the potential of *in vivo* infectivity of blood products containing the HAV genome must be assessed before destroying such material.

The choice of primers used in PCR amplification of viral RNA genomes after heat inactivation may also affect the relationship between PCR positivity and infectivity. Areas of the genome with a high degree of secondary structure will be more resistant to heating and ribonuclease digestion and should therefore be avoided. Amplification of small targets may result in positivity when very little of the genome is intact. In this study, it was shown that the whole genome remained intact by capturing the RNA at the 3 prime end and amplifying the 5 prime end.

These results also have implications for the use of nucleic acid amplification technology in the detection of other viruses which cannot be assayed in tissue culture, such as HCV and parvovirus B19, in virally inactivated blood products. It must be remembered that presence of PCR positivity does not necessarily indicate that the product contains infectious virus or even full length viral genomes.

Chapter 5

TIME COURSE OF HAV REPLICATION IN BS-C-1 CELLS

5.1 INTRODUCTION

The study of replication in picornaviruses has been facilitated by the rapid growth, high yields and cytopathogenicity, typical of members of this family. Poliovirus, the first of the picornaviruses to be propagated in cultured cells, replicates to produce yields of up to 10^5 PFU / cell in less than 10 hours of infection (Baltimore *et al.*, 1966). The typical single step growth curve of poliovirus consists of a lag phase, in which the virus attaches to the cell via a specific receptor, is internalised and uncoated, followed by a period of logarithmic replication until maximum virus titres are reached. During the lag phase, an eclipse of infectivity occurs as the viral RNA is released from the virus particles. The duration of this phase is usually 2-3 hr in poliovirus (Lwoff and Lwoff, 1961) and foot and mouth disease virus (Bachrach *et al.*, 1957). Rhinovirus 2 and 14 have a longer replication cycle, with a lag phase of 4-7 hr and maximal titres obtained at 10-18 hr post infection (Fiala and Kenny, 1967; Stott and Heath, 1970). The behaviour of HAV in cell culture markedly differs from that of other picornaviruses in that after 4-8 days of replication, persistent infection is generally established (De Chastoney and Siegl, 1987).

While the replication kinetics of the majority of picornaviruses may be comparable, the optimal conditions for maximum virus replication differs. The temperature at which infected cells are incubated particularly affects the yield of these viruses. Rhinoviruses 2 and 14, replicate optimally at 33-35 °C (Stott and Heath, 1970), whereas foot and mouth disease virus requires temperatures of 35-39.5 °C (Sharpe, 1958). Poliovirus replication is most efficient at 37 °C and is partially blocked at 39 °C (Lwoff and Lwoff, 1961).

Studies by Anderson *et al.* (1988) have suggested that the poor growth of HAV in tissue culture may be dependent on the efficiency of packaging of the HAV genome. These studies were based on a dot blot hybridisation method for RNA detection in which a single dilution series of the RNA was assayed be hybridisation with a radiolabelled probe. The amount of

RNA was quantitated by comparing the intensity of signal with known amounts of DNA from a plasmid containing the HAV sequence, detected by the hybridisation probe. While the dot blot method is quantitative, it does not take into account Poisson distribution as only a single sample at each dilution was hybridised. In addition, it also assumes that the sensitivity of detection of DNA by hybridisation is the same as that of RNA. Therefore the HAV genome copy number could not be directly correlated with the formation of infectious particles.

In the present study, the replication of HAV cytopathic strain HM175A.2 in BS-C-1 cells was assessed by comparing production and release of infectious virus particles with synthesis of HAV RNA using sensitive tissue culture and quantitative RT-PCR assays along with in *situ* hybridisation of viral RNA and immunofluorescent detection of antigen. The growth curve of strain HM175/18f which is less cytopathic in BS-C-1 cells was also determined by RNA quantitation and compared with that of HM175A.2. This study was initially designed to correlate RNA quantitation by RT-PCR with infectivity assay of HAV in order to investigate the value of nucleic acid based detection in determining the risk of transmission HAV via blood products. The quantitation of the HAV genome in this time course experiment is also useful in demonstrating the growth characteristics of HAV HM175A.2 and HM175/18f in BS-C-1 cells, although this does not strictly relate to the title of this thesis.

5.2 METHODS

5.2.1 Infection of BS-C-1 cells

Confluent flasks of BS-C-1 cells (passage 68) were split 1:2 into 12 culture flasks (25 cm^2) After 3 days incubation at 37 °C in buffered MEM containing 10 % FCS, cells from one flask were detached by treatment with 1 % trypsin as previously described and counted in a haemocytometer. Cells in 10 of the flasks were then inoculated with HAV HM175A.2 at a m.o.i. of 1 TCIU per cell. The culture medium was removed from 10 of the flasks and cells were washed with phosphate buffered saline. Cells were incubated with 0.5 ml virus at a

concentration of 1 x 10^7 TCIU ml^{-1} in buffered MEM 2 hr at 35 °C, 5 % carbon dioxide. A 9.5 ml volume of buffered MEM containing 2 % FCS was then added to the flasks. The remaining flask was mock infected with buffered MEM in exactly the same way. The time at which the virus was placed on the cells was designated 0 hr.

5.2.2 Harvesting cells and virus

At each time point (1-170 hr), a flask of infected cells was taken, 2 ml of medium was taken and stored at -70 °C . The cells were washed in PBS and detached by incubation with 4.8 % trypsin in PBS. The resuspended cells were washed twice in a total of 40 ml PBS and resuspended in 10 ml PBS. A 2 ml aliquot of the resuspended cells was subjected to 3 cycles of freezing at -20 °C and thawing at room temperature followed by centrifugation at 2600 x *g* to remove cellular debris. The supernatant was mixed with an equal volume of chloroform and centrifuged at 2600 x *g*. The resulting aqueous phase was stored at -70 °C. The remainder of the cell suspension was diluted to 2 x 10^5 cells / ml and cytospun onto ethanol washed microscope slides at 500 rpm for 5 min (250 µl /"cell spot"). After centrifugation, slides were air dried and fixed in 1 x Histochoice (Ameresco) for 30 min at room temperature. Fixed slides were washed in PBS and dehydrated through a series of 30 %, 50 %, 70 %, 90 % and 100 % ethanol, air dried and stored at -70 °C.

5.2.3 Immunofluorescence (IF)

Slides were taken from -70 °C to room temperature, allowed to equilibrate for 30 min and rehydrated through a series of 100 %, 90 %, 70 %, 50 % and 30 % ethanol. Affinity purified human post-convalescent serum diluted 1:250 in PBS was applied to each "cell spot" and incubated 4 hr at room temperature in a humidified chamber. Slides were then washed 3 times in PBS for 5 min. The secondary antibody, anti-human IgG, FITC conjugate (Sigma Chemical Co.) was diluted 1:20 in PBS, 0.005 % Evans blue and applied to the slides. After 30 min

incubation slides were washed as above and mounted in 10 % glycerol in PBS containing 0.25 % DABCO (Sigma Chemical Co.).

5.2.4 *In situ* hybridisation (ISH)

ISH was performed by Dr M. Taylor (St Mary's Hospital Medical School) on histochoice fixed cytospins of infected BS-C-1 cells using 30 base oligonucleotide probes as previously reported (Taylor *et al.*, 1992). Both antisense and sense polarity probes were used as shown in Table 5.1 The latter were used to examine the cells for the presence of replicative intermediates of the antigenomic HAV RNA. The probes were prepared on a Cyclone DNA oligonucleotide synthesizer (Biosearch Inc., New Brunswick Scientific Co., Inc., Edison, NJ) and 3' end-labelled to a specific activity of approximately $1x10^7$ cpm / pmol with dATP ^{35}S (Amersham, code SJ1334) using a commercial kit (NEP-100; NEN Du Pont, UK Ltd., Stevenage, UK) which uses a terminal deoxynucleotidyl transferase to add a polymeric tail of the radioligand to the 3' end of the probes. Throughout the *in situ* hybridisation procedure, only sterile diethyl pyrocarbonate-treated water or PBS and molecular biology grade chemicals (Sigma Chemical Co.) were used. Prior to hybridisation, slides were rehydrated by 2 min incubations in each of 100 %, 90 %, 70 %, 50 % and 30 % ethanol and washed for 5 min in PBS with gentle stirring. The slides were then blocked by incubation in 0.1 % acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min at room temperature with stirring followed by rinsing in water and dehydrating. Each cell spot was overlaid with 100 µl of hybridisation buffer consisting of 50 % deionized formamide (Fluka Chemie AG, Glossop, UK), 1 x Denhardt's solution (BSA, Ficoll and polyvinylpyrolidone, each at a final concentration of 0.02 %) 1 mM EDTA, 250 µg ml^{-1} denatured salmon sperm DNA, 10 % Dextran sulphate, 250 µg ml^{-1} yeast transfer RNA, , 250 µg ml^{-1} polyadenylic acid and 4 x sodium chloride / sodium citrate (0.6 M / 0.06 M). Before use, the hybridisation buffer was heated in a boiling water bath for 3 min and quenched on ice. DTT was added to yield a final concentration of 10 mM. Sections were overlaid with hybridisation fluid and incubated in a moist chamber for 3 hr at room

temperature. The oligonucleotide probes were diluted to give between 2.0 x 10^6 and 3.0 x 10^6 cpm / 100 µl of hybridisation fluid (1:5 dilution) and 50µl added to each prehybridised and drained 'cell spot'. Slides were incubated overnight in an air tight moist chamber. After hybridisation the slides were washed in 2 changes of 1 x SSC (150 mM sodium chloride, 15 mM Sodium citrate) at room temperature and 4 changes of 1 x SSC at 54-56 °C for 15 min each followed by a dip in water and air drying. Slides were then coated in 0.1 % chromalum, 0.01% gelatin, air dried and dipped in K5 nuclear track emulsion (Ilford Ltd, Mobberley, Cheshire, UK), diluted 1:1 in deionized H_2O containing 0.025% glycerol, and exposed in a light proof box for 19 days. Development was with Phenisol (Ilford Ltd.) followed by transfer to a acid stop bath (1 % acetic acid , 1 % glycerol), fixation in sodium thiosulphate and extensive washing in deionized water under dark room conditions. Slides were counter stained in Mayer's haemalum and mounted in DePex mounting medium (both from Merck Ltd., Lutterworth, Leicester, UK.). Slides were examined by conventional light microscopy and scored subjectively for intensity of hybridisation signal.

Table 5.1 Oligonucleotide Probes

Probe	Sequence	%GC
Antisense		
	1049 1380	
HAV-1	5'-CAG-TGA-AGT-ATA-AGC-TGA-AGT-TCC-TGT-CCC-3'	47
	3082 3053	
HAV-2a	5'-CTA-GGA-TCA-TCC-ACT-GAT-GAC-TCC-AGG-TCT-3'	47
	2371 2342	
HAV-3	5'-ATT-GTT-GTG-ATA-GCT-CCC-ACA-GGT-GCT-TGT-3'	47
Sense		
	3053 3082	
HAV-2s	5'AGA-CTT-GGA-GTC-ATC-AGT-GGA-TGA-TCC-TAG-3'	47
Controls		
	HAV-2 reverse antisense	
HAV-2ra	5'-TCT-GAA-CCT-CAG-TAG-TCA-CCT-ACT-AGG-ATC-3'	47
	Irrelevant 30-mer	
C1	5'-AGC-TAC-AAG-CAC-AAT-GGA-ACA-GAA-CTC-ACC-3'	47

5.2.5 HAV infectivity assay

Tissue culture fluid and lysed cells were assayed for infectious HAV by the cytopathic microtitre plate assay previously described. Briefly, 100 µl of 3 fold dilutions of each sample

in buffered MEM containing 2 % FCS were placed in wells of a microtitre plate (5 replicates per dilution). To each well was added 100 µl BS-C-1 cells (10^5 cells / ml) and plates were incubated at 35°C, 5 % CO_2. After 10 days incubation, 50 µl of buffered MEM containing 2 % FCS and 250 mM NaCl was added to each well and plates incubated a further 2 days prior to staining with Naphthalene black.

5.2.6 HAV RNA quantitation

RNA was extracted from tissue culture supernatant and lysed cells using a QIAmp viral RNA extraction kit (Qiagen Ltd.), reverse transcribed and amplified using primers A7a and A8a in the quantitative PCR method previously described (Chapter 2).

Infection of BS-C-1 cells with HAV HM175A.2 and subsequent harvesting and quantitative analysis was performed a total of 3 times on different days. All PCR and cytopathic microtitre plate assay results are shown as means. Flasks of BS-C-1 cells were also infected in exactly the same way with HAV HM175/18f (m.o.i. = 1 RFU ml^{-1}) and cells and media harvested as above for quantitative RT-PCR analysis.

5.3 RESULTS

5.3.1 Expression of HAV antigen in BS-C-1 cells

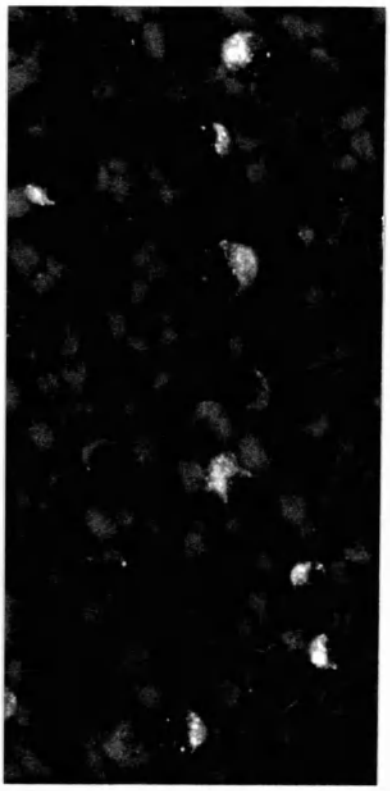
Infection of BS-C-1 cells was confirmed by immunofluorescent staining. Figure 5.1 is representative of the immunofluorescent staining seen throughout the time course of infection. Very little background was seen in the uninfected BS-C-1 cell control (Figure 5.1a) and a second negative control of infected cells incubated with diluted normal human serum instead of the primary antibody. HAV antigens were not detected until 40 hr post infection (p.i.) when approximately 10 % of cells were positive (Figure 5.1b). By 72 hr p.i., the majority of cells were expressing antigen at low levels.

Figure 5.1 Immunofluorescent staining of HAV HM175A.2 infected BS-C-1 cells

a) Mock infected BS-C-1 cells

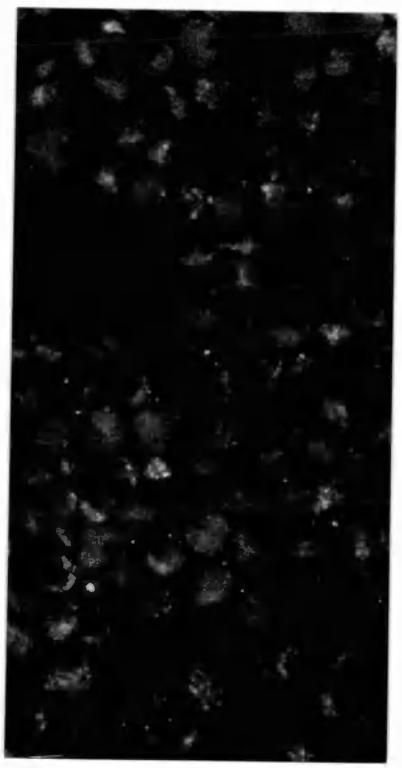
b-d)HAV HM175A.2 infected BS-C-1 cells

b)40 hr p.i.



c) 120 hr p.i.

d) 146 hr



Staining was most intense at 120 hr p.i. however, by 146 hr p.i., fewer cells expressed antigen (Figures 5.1c and 5.1d). Very few cells expressed antigen at 170 hr p.i..

5.3.2 Detection of HAV RNA by *in situ* hybridisation

5.3.2.1 Using antisense probes

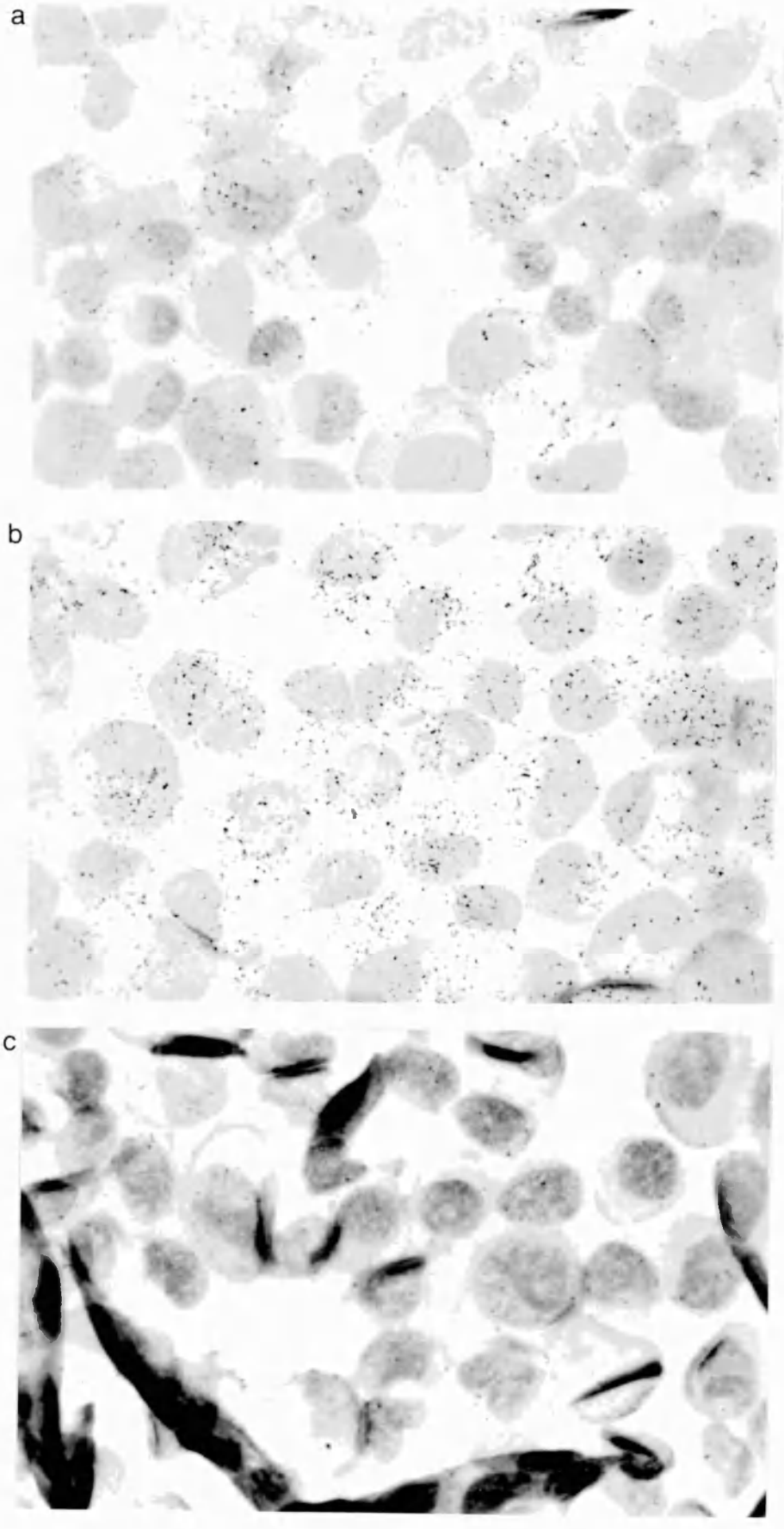
The presence of HAV RNA was only detected in cells from the 40 hr time point onwards. At this time, silver grains (20-50 grains / cell) were seen to be overlying approximately 3-4 cells per high power field. The distribution of this focal signal was predominantly cytoplasmic, although grains were also seen overlaying the nuclei. At 48 hr, the number of positive cells per high power field had risen to about 20. By 72 hr nearly all cells per field were positive (Figure 5.2a). No great change could be discerned on the 90 hr sample. By 120 hr all the cells remained positive and occasional cells showed more intense clusters of silver grains (80-120 grains / cell). Maximum hybridisation signal was seen on the 146 hr sample where 9 / 10 cells exhibited similar intense labelling with the antisense probes (Figure 5.2b). The pattern of signal described was representative of 3 separate timecourse experiments. Appreciable cell loss was frequently associated with the last time point at 170 hr. These cells were lost during the high stringency washes at 56 °C in 1 x SSC. No significant cell loss was noticed from slides at the other time points. Cells which remained on the 170 hr slides showed only sporadic silver grains. In contrast, very few background grains were seen in uninfected cells (Figure 5.2c)

5.3.2.2 Using sense probes

No specific hybridisation signal was found at any of the time-points when sense polarity probes were used.

Figure 5.2 ***In situ*** **hybridisation of HAV HM175A.2 infected BS-C-1 cells**

a) HAV HM175A.2 infected BS-C-1 cells - 72 hr p.i., b) 146 hr p.i., c) Mock infected BS-C-1 cells



5.3.2.3 Control experiments

The ISH method has previously been fully validated on both cryostat and paraffin embedded tissue sections (Taylor *et al.*,1992, Taylor *et al.*, 1994). In the present study using cytospins, specificity was again confirmed by prior digestion with RNases A and T1. This treatment abolished hybridisation.

5.3.3 Cell and culture medium associated infectious HAV particles

Variations in levels of released and cell associated infectious virus throughout the timecourse are shown in Table 5.2 and Figure 5.3 and total infectious virus titres in Figure 5.4. All data were adjusted to give titres per 10^6 cells for ease of comparison. Cells were infected at a multiplicity of infection of 1 TCIU per cell (6 $\log_{10}$ TCIU / 10^6 cells). After 1 hr virus adsorption, the titre of infectious virus in the cell culture medium was 4.88 $\log_{10}$ TCIU / 10^6 cells. Thus approximately 90 % of virus was taken up by the cells during the first hour (Figure 5.3 -■-). At 16 hr p.i., only 2 % of the initial inoculum was recovered in the culture medium. The titre of infectious HAV virions released into the cell culture medium increased logarithmically from 4.27 $\log_{10}$ TCIU / 10^6 cells at 16 hr to 6.93 $\log_{10}$ TCIU / 10^6 cells at 48 hr p.i. (Figure 5.3 -■-). The rate of viral replication then began to decrease. By 72 hr, a stationary phase was entered (7.24 $\log_{10}$ TCIU / 10^6 cells. The mean titre obtained in the stationary phase was 7.31 $\log_{10}$ TCIU released / 10^6 cells (~20 TCIU released / cell). In this 'stationary phase' no further increase in total HAV titre was detected, however, cells remained viable and CPE was not seen. The growth curve for cell associated virus followed the same pattern as that of released virus with a mean titre of 7.28 $\log_{10}$ TCIU / 10^6 cells (~20 cell associated TCIU / cell) during the stationary phase (Figure 5.3 -□-). The total amount of HAV produced per cell during the timecourse was approximately 40 TCIU / cell.

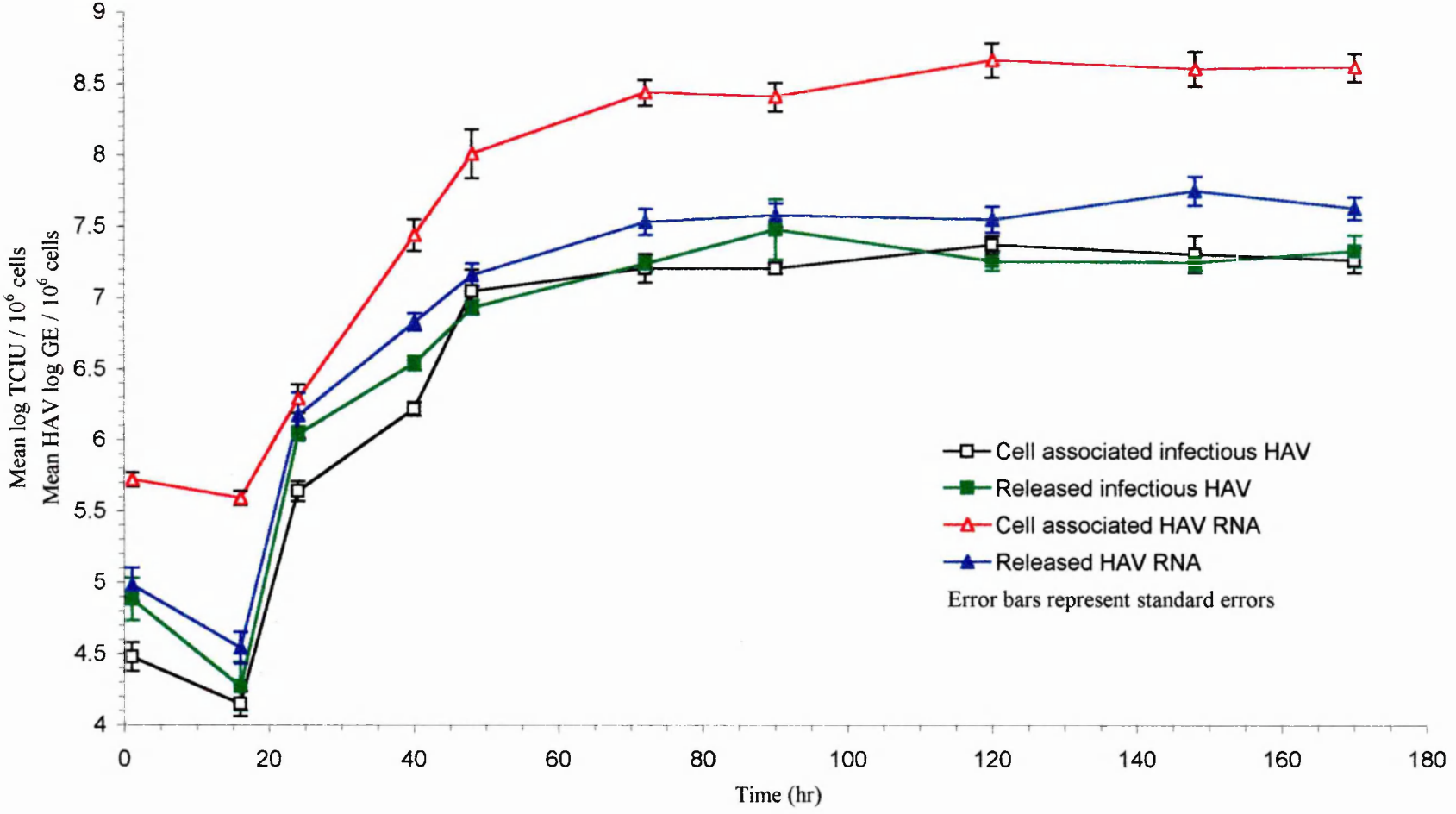


Figure 5.3. Accumulation and release of HM175A.2 infectious particles and genome in B-SC-1 cells

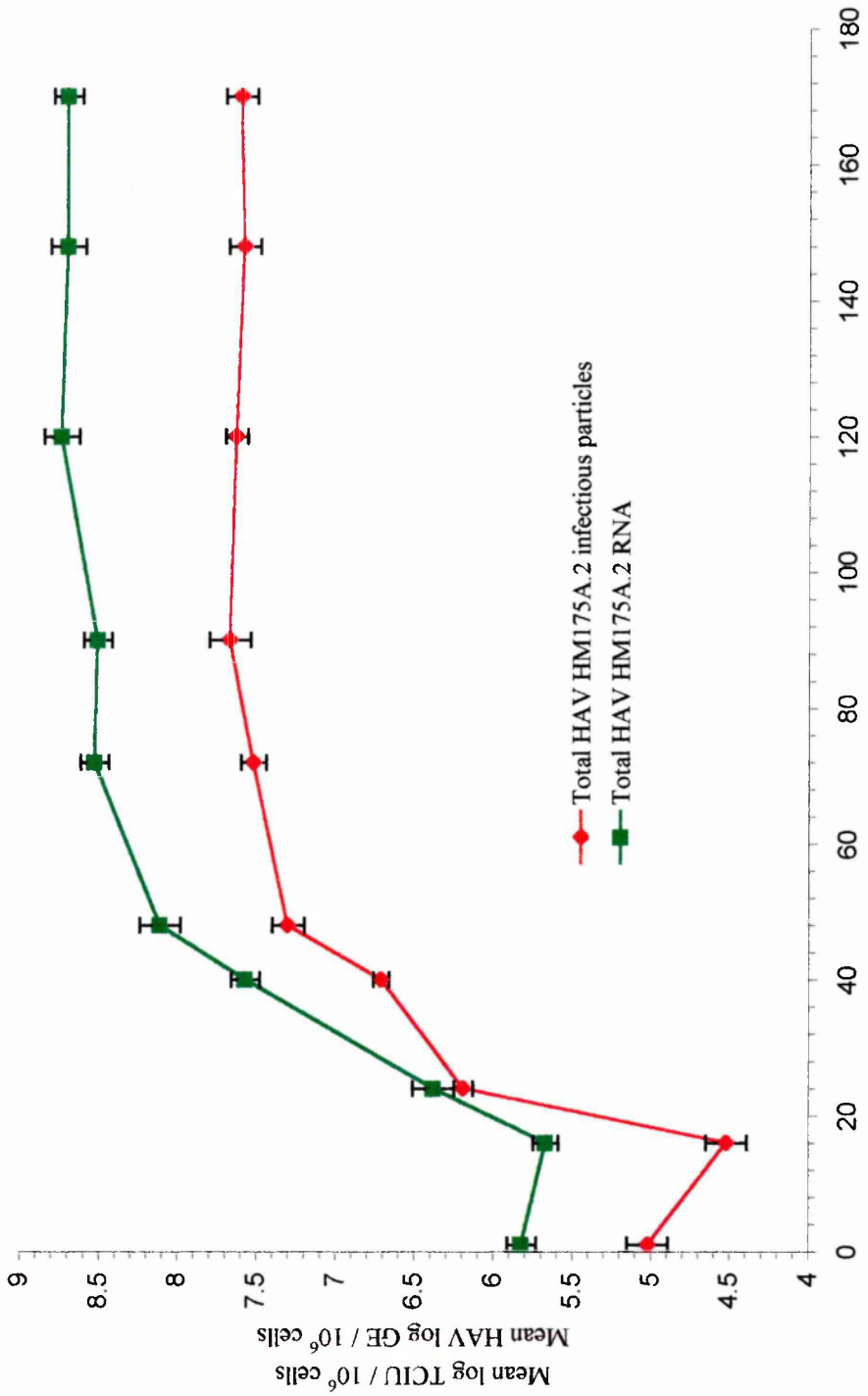


Figure 5.4 Total HAV HM175A.2 infectious particles and genome in B-SC-1 cells

Table 5.2. Levels of cell and culture medium associated HAV HM175A.2 tissue culture infectious particles following infection of BS-C-1 cells

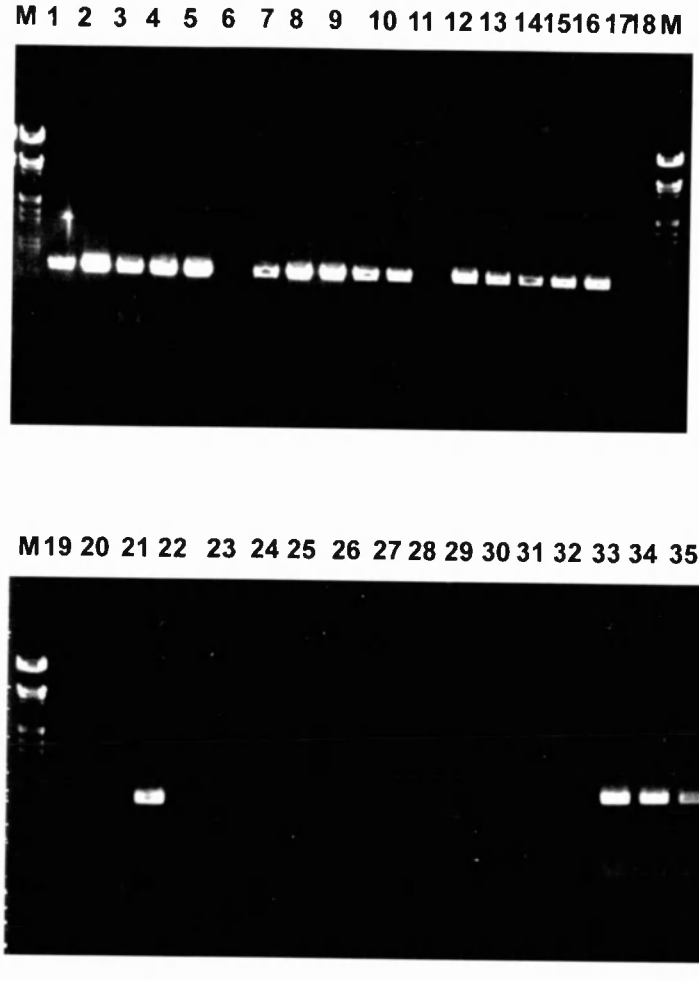
Time	Cell associated infectious HAV HM175A.2 (TCIU / 10^6 cells)					Medium associated infectious HAV HM175A.2 (TCIU / 10^6 cells)					
(Hr)	1	2	3	Mean	S. E.	1	2	3	Mean	S. E.	Total
1	4.35	4.66	4.43	**4.48**	0.10	5.15	4.67	4.83	**4.88**	0.15	**5.02**
16	4.30	4.00	4.16	**4.15**	0.09	4.52	3.95	4.35	**4.27**	0.17	**4.52**
24	5.63	5.53	5.77	**5.64**	0.07	5.94	6.10	6.08	**6.04**	0.05	**6.19**
40	6.23	6.30	6.13	**6.22**	0.05	6.56	6.61	6.46	**6.54**	0.05	**6.71**
48	7.23	6.75	7.17	**7.05**	0.15	6.88	6.87	7.03	**6.93**	0.05	**7.30**
72	7.39	7.16	7.06	**7.21**	0.10	7.29	7.30	7.12	**7.24**	0.06	**7.52**
90	7.29	7.15	7.20	**7.21**	0.04	7.89	7.27	7.28	**7.48**	0.21	**7.67**
120	7.38	7.49	7.27	**7.38**	0.06	7.36	7.30	7.12	**7.26**	0.07	**7.63**
148	7.57	7.22	7.14	**7.31**	0.13	7.31	7.13	7.32	**7.25**	0.06	**7.58**
170	7.20	7.16	7.45	**7.27**	0.09	7.54	7.18	7.26	**7.33**	0.11	**7.60**

5.3.4 Cell and culture medium associated HAV RNA

The release of HAV RNA from BS-C-1 cells during the timecourse was similar to that of infectious particles (Table 5.3, Figure 5.3). The levels of RNA in the culture fluid increased logarithmically from 4.54 $\log_{10}$ genomes / 10^6 cells at 16 hr to 7.16 $\log_{10}$ genomes / 10^6 cells at 48 hr. As seen with the levels of infectious virus, at 72 hr, a stationary phase was entered. The mean titre of HAV RNA detected in the cell culture medium was 7.61 $\log_{10}$ genomes / 10^6 cells (~41 genomes released / cell, Figure 5.3 -▲-).

The titres of cell associated HAV RNA were higher than the corresponding titres of cell associated infectious virus particles, although the patterns of the time courses were similar (Figure 5.3). A logarithmic increase in cell associated RNA was seen from 5.59 $\log_{10}$ genomes / 10^6 cells at 16 hr to 8.44 $\log_{10}$ genomes / 10^6 cells at 48 hr. The mean titre of cell associated HAV RNA during the stationary phase (72 - 170 hr) was 8.59 $\log_{10}$ genomes / 10^6 cells (~355 genomes / cell, Figure 5.3 -Δ-). At the stationary phase, combined titres of cell associated and released HAV give a total of ~396 genomes / cell. Figure 5.4 shows the production of total HAV infectious virus and total HAV RNA.

Figure 5.5 Example quantitative RT-PCR assay of cell associated HAV HM175A.2



Samples (dilutions of cell associated HAV RNA 1 hr post infection)

M	molecular weight markers 1		
1-5	cDNA diluted 1:3	19-23	cDNA diluted $1:10^2$
6	negative control water	24	Negative control water
7-11	cDNA diluted 1:10	25-29	cDNA diluted $1:10^{2.5}$
12	negative control water	30-32	Negative control water
13-17	cDNA diluted $1:10^{1.5}$	33+34	Standard CS101 1:10
18	negative control water	35+36	Standard CS101 1:100

Calculation of HAV RNA titre

Result of GLIM analysis = 50 genomes / sample

log RNA titre / 10^6 cells = $\log (50 \times (100/8.8)^a \times (1000/3.5)^b \times 3.33^c)$

= **5.73 log genomes ml^{-1}**

[a] Replication efficiency factor
[b] Volume factor (1ml)
[c] Cell concentration = 3×10^5 / ml

Although the curves are similar, a t-test of the mean data taken at the stationary phased showed that levels of total RNA are significantly higher ($P<0.01$) than those of infectious virus. Levels of total RNA were 10 fold higher than infectious virus during this phase.

Table 5.3. Levels of cell associated and released HAV HM175A.2 RNA following infection of BS-C-1 cells

Time	Cell associated HAV HM175A.2 RNA (genomes / 10^6 cells)					Medium associated HAV HM175A.2 RNA (genomes /10^6 cells)					
(Hr)	1	2	3	Mean	S. E.	1	2	3	Mean	S. E.	Total
1	5.73	5.80	5.62	**5.72**	0.05	4.75	5.05	5.13	**4.98**	0.12	**5.97**
16	5.62	5.49	5.66	**5.59**	0.05	4.49	4.75	4.39	**4.54**	0.11	**5.62**
24	6.21	6.48	6.18	**6.39**	0.10	5.88	6.40	6.23	**6.17**	0.16	**6.54**
40	7.24	7.46	7.62	**7.44**	0.11	6.95	6.79	6.74	**6.83**	0.06	**7.54**
48	7.68	8.16	8.19	**8.01**	0.17	7.25	7.24	7.00	**7.16**	0.08	**8.07**
72	8.26	8.56	8.50	**8.44**	0.09	7.35	7.63	7.62	**7.53**	0.09	**8.49**
90	8.25	8.37	8.61	**8.41**	0.10	7.48	7.74	7.53	**7.58**	0.08	**8.47**
120	8.88	8.48	8.65	**8.67**	0.12	7.38	7.68	7.59	**7.55**	0.09	**8.70**
148	8.46	8.54	8.83	**8.61**	0.12	7.92	7.56	7.78	**7.75**	0.10	**8.67**
170	8.80	8.46	8.60	**8.62**	0.10	7.49	7.75	7.64	**7.63**	0.08	**8.66**

5.3.5 Assay of HAV HM175/18f RNA

The accumulation of HAV HM175/18f RNA in BS-C-1 cell can be seen in Figure 5.6. Each data point represents the mean of 3 experiments. During the first 16 hr post infection, levels of HAV RNA in the medium decreased from 6.2 $\log_{10}$ genomes / 10^6 cells to 5.14 $\log_{10}$ as virus was taken up into the cell while conversely, an increase in the cell associated RNA to 5.5 $\log_{10}$ genomes / 10^6 cells was seen. By 24 hr, a small increase in the level of viral RNA was seen in the culture medium and HAV continued to be released until at 170 hours post infection the titre reached 7.63 $\log_{10}$ genomes / 10^6 cells (~ 42 genome equivalents / cell). The titre of cell associated RNA also continued to rise at a logarithmic rate until 72 hr post infection (7.97 $\log_{10}$ genomes / 10^6 cells). The rate of viral RNA accumulation in the cells then decreased and a stationary phase was entered. Levels of RNA associated with the cells were generally higher than that in the medium, reaching a maximum of 8.30 $\log_{10}$ genomes / 10^6 cells at 170 hr post infection (~200 genome equivalents / cell)

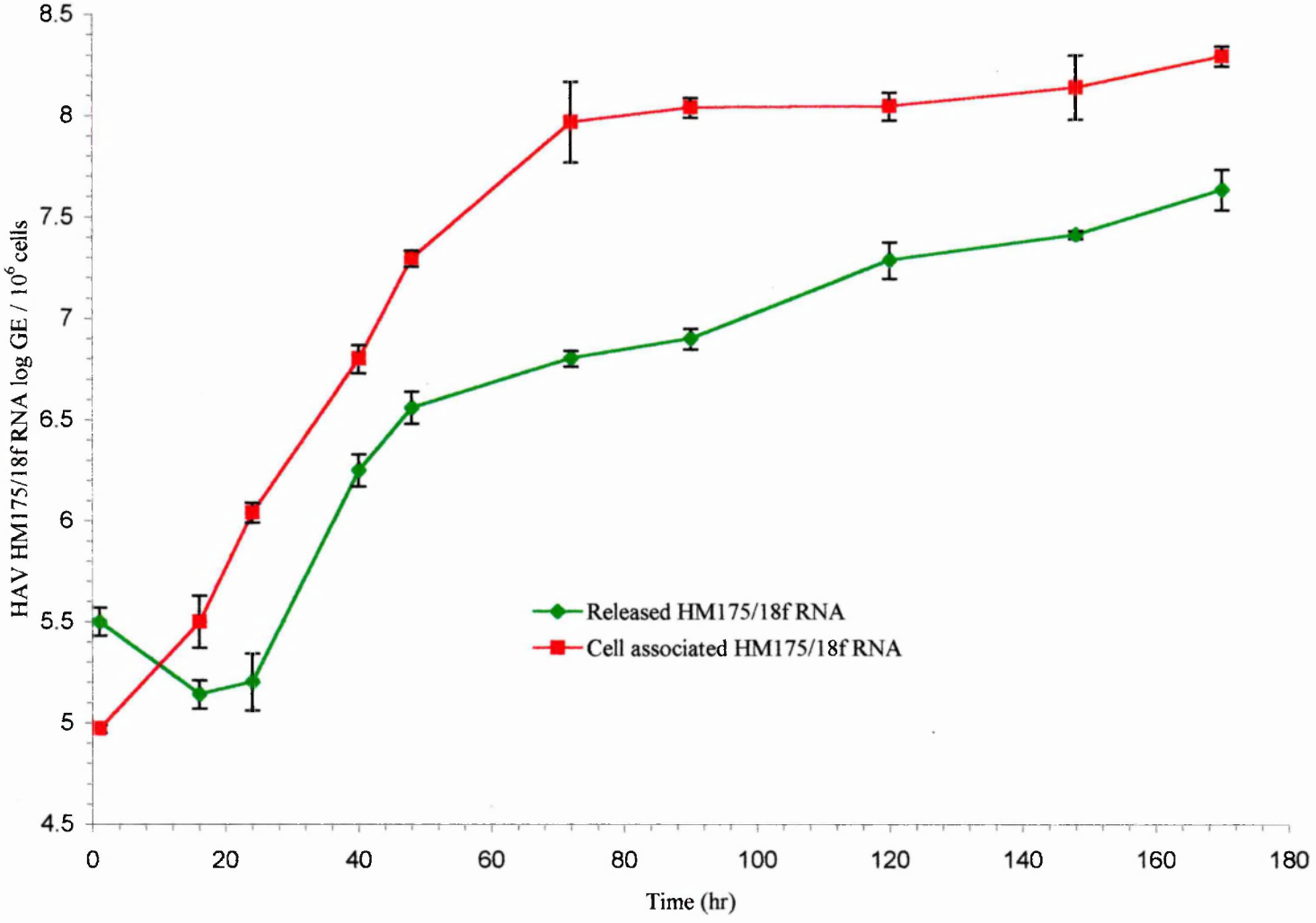


Figure 5.6 Accumulation and release of HM175/18f RNA in B-SC-1 cells

5.4 DISCUSSION

Several studies into the kinetics of HAV replication have been carried out using various cytopathic strains (Anderson *et al.,* 1987; Anderson *et al.,* 1988; Cromeans *et al.,* 1989) However, where levels of RNA have been measured, the results given have not been in terms of genome copies and therefore could not be directly compared with yields of infectious virus. In this study, sensitive RT-PCR assay for HAV RNA gave results in a form which could be directly related to that of the cytopathic microtitre plate assay for infectivity as they are both based on limiting dilution analysis. As HM175/18f did not give a good CPE in BS-C-1 cells, it was not possible to obtain reliable data in the time course of infectious virus for this variant.

In order to obtain results that could be directly compared with those of Anderson and co-workers (1987; 1988), cells were infected at a multiplicity of infection of 1. However, at this concentration, it cannot be guaranteed that all cells will become infected simultaneously due to Poisson distribution. While it is possible that infection at a higher multiplicity of infection may have resulted in earlier production of virus, in a similar study, Harmon *et al.* (1989), showed that infection of cells at a multiplicity of infection of both 10 and 1 RFU/cell resulted in similar growth curves. Inoculation at 0.1 RFU/cell resulted a 2-3 day lag in the appearance of virus.

The first event in HAV infection of BS-C-1 cells is the uptake and uncoating of the virus. In the present study, during the absorption phase, the total amount of infectious virions present in the medium decreased by 90 % (from 6 to 4.88 $\log_{10}$ TCIU / 10^6 cells) and after 16 hr, only 2 % (4.27 $\log_{10}$ TCIU / 10^6 cells) of the initial inoculum was detected by cytopathic assay. The eclipse phase of HAV in BS-C-1 cells is therefore comparable that of poliovirus in which 90 % of virus is eclipsed by 2 hr p.i. (Anderson *et al*, 1988). However with poliovirus, the production of progeny virus can be seen 2-4 hr p.i. whereas progeny virus was not detected in BS-C-1 cells with HAV HM175A.2 until 24 hr p.i. (Figure 5.3). The low levels of infectious virus

associated with the cell at 1 and 16 hr p.i. compared with levels of RNA tends to suggest that the majority of virus was quickly uncoated upon cell entry. It has been suggested extended lag phase of HAV compared with poliovirus may be due to asynchrony in the uncoating of HAV infection (Wheeler *et al.*, 1986; Cromeans *et al.*, 1989; Cho and Ehrenfeld, 1991). However, Anderson *et al.* (1987) showed that penetration and uncoating of variant HM175A occurred at a similar rate to that of poliovirus. These data along with the results of this study suggest that the replication of HAV is not restricted by slow or asynchronous uncoating but rather at a later stage in infection The rate at which cytopathic variant HM175A.2 is rendered non-infectious by uncoating in BS-C-1 cells appears to a little higher than previously reported for HAV (Anderson *et al.*, 1987) in which 25 % of the closely related strain HM175A was still recovered up to 9 hr p.i.. HAV strain HM175A.2 is a large plaque forming variant of HM175A which was clonally selected and acutely passaged 4 times. The ability of this variant to form large plaques may be related to the faster uptake of the virus by BS-C-1 cells.

Since no samples were taken between 1 and 16 hours or 16 and 24 hours, information regarding the initial stages of HAV infection may have been lost. For example, the titre of the virus in the supernatant may have been further reduced by cell entry than has been recorded either before or after the 16 hour time point. A more detailed study of early events occurring in the infection of cells with HAV would require the assay of more samples between the first and twenty fourth hours of infection. The fact that only 10 % of the cells expressed levels of viral antigen or RNA that could be detected by *in situ* methods also suggests that this is not a single step growth curve.

Following the lag phase, HAV accumulated logrithmically until 48 hr p.i. and by 72 hr p.i., reached total mean titre of 40 infectious particles / cell. The yield of this virus is higher than the value of 20 RFU/cell observed by Anderson *et al.* (1987) although the kinetics of replication appear to correlate well. The increase in viral yield may be a result of the more efficient virus

uncoating seen with this variant. More efficient uncoating of HAV and subsequently higher viral yield may be responsible for improved development of cytopathic effects.

Throughout the timecourse, levels of HAV RNA released into the culture fluid closely resembled those of infectious virus. However, statistical analysis of the mean titres obtained during the stationary phase showed significant difference between released infectious virus and viral RNA at the 95 % confidence level. Approximately 50 % of HAV genomes released during this stationary period were capable of infecting BS-C-1 cells (20 released TCIU / cell, 42 released genomes / cell). It is expected that all viral RNA detected would be encapsidated as any naked RNA would be quickly degraded once released into the tissue culture fluid. Therefore, a proportion of HAV RNA appears to be released as non-infectious particles which may correspond to the RNA containing provirions detected by Ruchti *et al.* (1991) or correctly encapsidated, defective RNA genomes. The ratio of PCR detected RNA molecules to TCIU released into the tissue culture medium during the stationary phase was found to be approximately 2:1. Based on cDNA-RNA hybridisation studies, Jansen *et al.* (1988) found a tissue culture extracted preparation of HAV strain HM175p16, which had been adapted to rapid, non-cytopathic growth in BS-C-1 cells, to contain approximately 58 genomes per RFU whereas a preparation of the wild type HM175 contained 2.4×10^5 genomes per RFU.

The ratio of RNA to infectious virus (58:1) reported by Jansen and co-workers for the tissue culture adapted strain of HAV, p16 HM175 (non-cytopathic), was determined after CsCl density gradient purification of virus from the lysate obtained after freezing and thawing infected cells. This figure should therefore be compared with the ratio of cell associated HAV genome to cell associated infectious virus. The mean level of cell culture infectious HAV HM175A.2 in the stationary phase was 7.28 log TCIU / 10^6 cells while that of the viral genome was 8.55 log genomes / 10^6 cells. Therefore the ratio of Cell associated HAV genome to infectious virus was 19:1. Jansen and co-workers showed that after 16 passages in cell culture,

the genome to infectivity ratio of HAV HM175 was reduced from 2.4 $x10^5$ genome copies per RFU to 58 genome copies per RFU. The cytopathic strain, HAV HM175A.2 is derived from the wild type virus after 30 passages of persistently infected cells, and 8 passages of the resulting virus followed by 3 rounds of plaque purification and 4 further passages (Anderson 1987). The high level of adaptation to tissue culture achieved may also have resulted in a further reduction in the genome to infectivity ratio.

Levels of HAV remaining associated with BS-C-1 cells during the stationary phase were similar to those of released virus. Therefore, approximately 50 % of all infectious virions were released. This percentage of released virus is higher than previously noted with non-cytopathic strains of HAV (Siegl *et al.,* 1984; Nasser and Metcalf, 1987) but correlates well with data obtained with an alternative HM175 derived cytopathic variant (Cromeans *et al.*, 1989). The release of infectious virus may be either a result of cytopathology or a factor involved in the ability of cytopathic strains to cause cell death.

The amount of HAV RNA remaining associated with the cells throughout the timecourse was generally 10 fold higher than that of cell bound infectious virus. During the stage of virus adsorption and uncoating, the majority of HAV RNA was taken up into the cells and only a small percentage was degraded by 16 hr p.i.. However, the logarithmic rate of infectious HAV replication between 16-24 hr was double that of viral RNA replication (Figure 5.4). By 24 hr p.i., approximately 44 % of viral RNA was detected as infectious virus particles. Since the ratio of packaged RNA to infectivity was estimated at 2:1, the actual percentage of RNA which is packaged at this stage may be as high as 90 %. This supports the theory of Anderson *et al.* (1988) that during the early stages of HAV infection in cell culture, the levels of RNA are low due to highly efficient encapsidation. The subsequent increase in the pool of non-encapsidated RNA may be due to an alteration in processing of the HAV polyprotein caused by the high excess of pentamers during the earlier stages of replication. Borovec and Anderson (1993)

suggested that cleavage of the polyprotein tends to occur in *trans* when pentamer levels are high, resulting in the formation of 5 S monomers which cannot assemble into virions.

Both immunofluorescent staining of HAV antigen and ISH demonstrated the asynchronous nature of HAV replication previously reported. The percentage of cells expressing HAV RNA and antigen increased over time. At 40 hr only 10 % of cells were expressing viral antigen or RNA apparently at differing levels, however, by 72 hr, viral transcription and translation was demonstrated in the majority of cells. These results are similar to those seen by Harmon *et al* (1989) who used the same *in situ* techniques to study the replication of a non-cytopathic stock of HAV HM175 (passage 30). This non-cytopathic virus replicated very slowly reaching maximum yields of RNA at 8 days p.i. and only showing antigen expression in all cells after 14 days. Although asynchrony may be partly responsible for the protracted replication of HAV, it appears that the final yield and cytopathic effect are not influenced. It is possible that the low titre the HAV inoculum may have been responsible for the apparent asynchronous replication, however, Harmon and co-workers showed comparable results after infection of B-SC-1 cells with HAV at titres of both 1 and 10 RFU / cell.

Levels of antigen expressed in infected BS-C-1 cells appear to decline by 146 hr when both HAV RNA and infectious virus titres are still at a maximum. This may be due to the loss of fragile infected cells from the slides during washing leaving only cells expressing small amounts of antigen.

The sensitivity of the ISH method used was determined from the RNA titre determined by quantitative PCR and the staining seen in cells at 40 hr. The number of genomes required for ISH detection was estimated at approximately 28 copies per cell. Negative strand HAV RNA could not be detected using sense polarity probes in ISH. Assuming that the sense polarity probes were of the same sensitivity as the anti-sense probes, it may be concluded that levels of the replicative intermediate did not reach 28 RNA copies per cell throughout the timecourse.

When the replication of poliovirus was studied in HeLa cells, it was estimated that 5-10 % viral RNA was in the negative strand form (Baltimore and Girard, 1966). Since levels of positive strand HAV RNA were only estimated to be 355 genomes / cell it is not surprising that the replicative intermediate was not detectable by ISH.

RNA replication determined by the quantitative PCR method showed good correlation with the infectivity growth curve. Therefore the method was also used to compare the growth of the variant HM175A.2 with the less cytopathic variant HM175/18f. During the initial stages of infection, levels of HM175/18f RNA in the medium decreased as expected. However, the degree to which this variant was removed from the culture medium by cell entry was less than that of HM175A.2. After the initial hour post infection, 25 % of the initial RNA titre was still detected in the culture medium and only 90% of the virus appeared to have entered the cells at 16 hr, after which released progeny virus was detected by RT-PCR (Figure 5.6).

After 24 hr, HM175/18f RNA accumulated in BS-C-1 cells at in a similar manner to that of the more cytopathic variant, with a logarithmic growth phase from 24 - 72 hr followed by a stationary phase (Figure 5.7). However, the rate of RNA replication is slower and maximum levels of viral RNA in the cells is lower than that of HM175A.2. Between 24 and 48 hr p.i., the rate of release of viral RNA paralleled that of increase in cellular levels whereas later in infection, the rate of increase is slower. In the early stages of HAV replication efficient packaging of RNA may result in the release of virus particles at the same rate as RNA accumulation. The polyprotein processing mentioned above may subsequently restrict viral packaging and therefore result in an accumulation of non-packaged RNA which is not released. The lower rate of release of HM175/18f continues while viral RNA replication appears to be in the stationary phase. Levels of released HM175/18f RNA released into the medium eventually rise to a similar level to that of released HM175A.2. RNA.

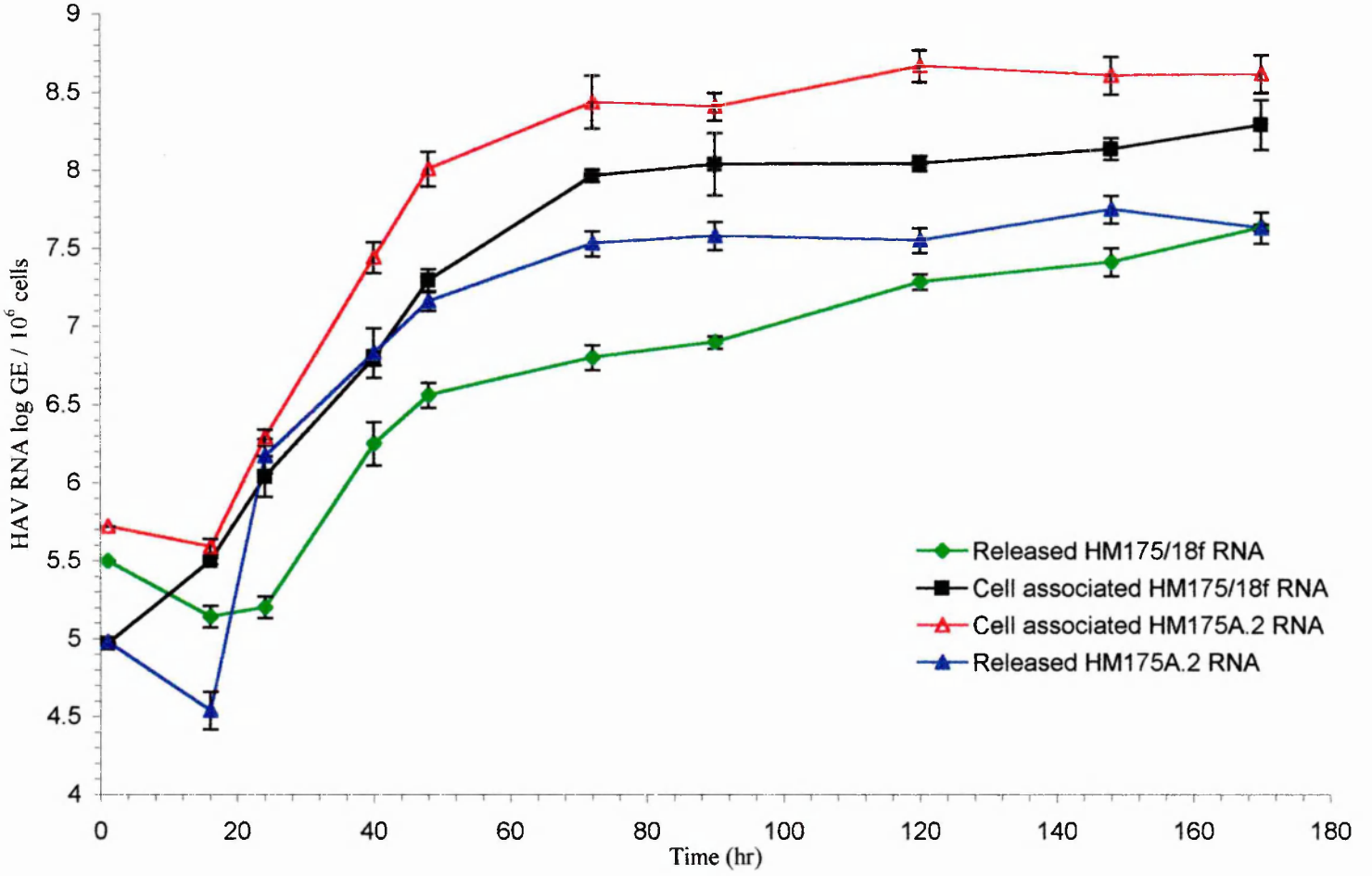


Figure 5.7 Comparison of RNA replication of HAV HM175A.2 and HM175/18f in B-SC-1 cells

Chapter 6

REPLICATION OF WILD TYPE AND CELL CULTURE ADAPTED STRAINS OF HAV IN A TAMARIN MODEL

6.1 INTRODUCTION

Passage of HAV in cell culture has led to attenuation of virulence in marmosets and chimpanzees (Provost *et al.*, 1982, Bradley *et al.*, 1984; Karron *et al.*, 1988 and Taylor *et al.*, 1993). In this study, the virulence of two cell culture adapted variants of HAV HM175 were compared with the wild type virus in a tamarin model. The variant HM175/18f is cytopathic in FRhK-4 cells but shows limited cytopathology in BS-C-1 cells whereas HM175A.2 has been adapted to cytopathic growth in BS-C-1 cells. The quantitative RT-PCR assay was used in order to directly compare faecal shedding and viraemia between the animals inoculated with the various HAV preparations. Replication of the virus in the liver was also demonstrated by immunohistochemistry. The extent of liver damage was determined by histological analysis and measurement of serum alanine amino transferase (ALT) levels which become elevated upon disruption of the liver. An enzyme linked immunosorbant assay was used to show seroconversion. Faecal and serum samples from animals inoculated with strain HM175A.2 were also analysed by the cytopathic microtitre plate assay for HAV.

6.2 METHODS

6.2.1 Inoculation of animals

The progression of infection of 3 variants of HAV strain HAV HM175 was studied in red bellied tamarins (Sanguinus labiatus). Three groups of 3 tamarins were intravenously inoculated with 0.1 ml of HAV HM175 wild type (obtained from Dr Purcell, National Institute of Health, Bethesda, Maryland, USA) or the tissue culture adapted variants HM175/18f and HM175A.2 (Table 6.1) by staff in the Biological Services Section at NIBSC. Each of the virus preparations were diluted to approximately 10^5 genomes ml^{-1} in 6 salt PBS containing 10 % FCS. The 3 groups of tamarins were selected to give the same distribution of males and females in each group and similar mean group weights.

Table 6.1 Tamarin inoculation groups

Group	Tamarin	Sex	Weight (g)
1: **HAV HM175 wt**	C9	M	540
	C16	F	615
	C20	M	500
2: **HAV HM175/18f**	C13	M	612
	C17	F	615
	C19	M	425
3: **HAV HM175A.2**	C12	M	530
	C15	F	540
	D3	M	555

6.2.2 Collection of samples

Prior to inoculation and until day 70 post inoculation, blood, faeces and needle liver biopsies were taken for analysis as shown in Table 6.2.

Table 6.2. Collection of samples from tamarins

Day	Bleed	Faecal collection	Liver biopsy
-28	✓	✓	✗
-13	✓	✓	✓
0	✓	✓	✗
4	✓	✓	✗
9	✓	✓	✗
14	✓	✓	✗
17	✓	✓	✗
21	✓	✓	✓
28	✓	✓	✗
35	✓	✓	✗
42	✓	✓	✗
49	✓	✓	✗
56	✓	✓	✗
63	✓	✓	✗
70	✓	✓	✓

On the day of collection, 1 g of faeces was weighed and placed in a universal tube and 10 ml MEM + 5 % bicarbonate was added. A faecal suspension was made by adding 2 ml sterile glass beads (BDH) and vortex mixing. The suspension was then centrifuged at 1300 x *g* for 10 min at 4 °C. The supernatant was transferred to a sterile polypropylene tube. A

1 ml volume of supernatant was placed in a 2 ml screw capped microtube and extracted with an equal volume of chloroform. After mixing the sample with chloroform, it was centrifuged at 13400 x *g* for 5 min. The aqueous layer was transferred to a new microtube and stored at -70 °C. The remainder of whole faeces and non-extracted supernatant were stored at -20 °C.

Tamarin blood samples (0.5-1 ml) were taken and stored in 2 ml microtubes at 4 °C for 12-16 hr allowing clotting to occur. Samples were then centrifuged at 13400 x *g* for 20 min at 4 °C. The supernatant was transferred to a clean microtube and centrifuged a further 20 min as above. The supernatant was stored in 2 ml microtubes at -70 °C.

6.2.3 HAV RNA quantitation

RNA was extracted from all faecal suspensions and serum samples using QIAmp viral RNA extraction kit (Qiagen Inc.), reverse transcribed and amplified using primers A7a and A8a in the quantitative PCR method.

6.2.4 Infectivity assay for HAV HM175A.2

Faecal and serum samples from tamarins infected with HAV HM175A.2 were diluted 1:10 followed by 0.5 log dilutions and analysed by the cytopathic microtitre plate assay.

6.2.5 Detection of antibodies to HAV in tamarin sera

As time did not permit the use of the antibody neutralisation assay described in chapter 3, serum samples were assayed for an immune response to HAV using the commercial kit Murex HAV VK38 (Murex Biotech Ltd, Dartford, UK) which detects total antibodies to HAV by enzyme immunoassay. In this assay, the test sample is incubated with peroxidase conjugated anti-HAV in a HAV coated well. Any antibodies to HAV in the sample

compete with the peroxidase conjugate in binding to the antigen in the plate. After washing, the peroxidase activity is measured by the addition of a chromogen which develops a blue colour which can be measured by spectrophotometry at 450 nm in proportion to the amount of peroxidase conjugate present. If the test sample contains antibodies to HAV, the $O.D._{450}$ will be reduced. The assay was performed according to the manufacturers instructions. A 96 well microtitre plate which had been coated with formalin inactivated, heat treated human HAV (supplied in the kit) was taken from 4 °C to room temperature. Leaving the first well (A1) blank as a substrate control, 10 µl of a negative control was dispensed into each of 3 wells and 10 µl of the positive control into a further 2 wells. The remainder of the wells were used to assay 10 µl of each of the tamarin serum samples. A 200 µl volume of conjugate was then placed in each well except A1 and the edge of the plate tapped to mix the conjugate with the samples. The plate was sealed with an adhesive cover and incubated at 37 °C for 3 hr. The contents of each well was then removed by aspiration and wells were washed four times in the supplied wash buffer. The plate was blotted onto absorbent tissue and 200 µl of chromogen / substrate was added to each well. After a 30 min incubation at room temperature, 50 µl of stop solution was added in the same sequence and time intervals as for the chromogen/substrate. The plate was read on a spectrophotometer at 450 nm using well A1 as a blank. In order to determine the validity of the test, the mean $O.D._{450}$ of the 2 positive control wells was subtracted from that of the negative control wells. For the assay run to be valid, this value must be greater than 0.3. A cut-off value for determining positive samples was obtained by dividing the sum of the mean positive and negative absorbencies by 2. All samples with readings lower than or equal to the cut off were positive for anti-HAV and those with a higher reading were considered negative.

6.2.6 Assay of serum alanine amino transferase levels

Each serum sample was measured for ALT levels using the commercial kit ALT 10 (Sigma Diagnostics, St Louis, MO, USA). In this assay, the sample is incubated with L-alanine, 2-oxoglutarate, nicotinamide adenine dinucleotide (NADH) and lactate dehydrogenase (LD). Any ALT present in a sample catalyses the conversion of L-alanine and 2-oxoglutarate to pyruvate and L-glutamate as shown in Figure 6.1. The lactate dehydrogenase then causes the reduction of pyruvate to lactate and simultaneous oxidation of NADH to NAD. NADH absorbs light at 340 nm and conversion to NAD causes a decrease in the absorbance at this wavelength. The rate of decrease in absorbence at 340 nm in this assay is directly proportional to the ALT activity of the sample.

Figure 6.1 Enzymatic reactions involved in the measurement of ALT levels

$$L-Alanine+2-oxoglutarate \xrightarrow{ALT} Pyruvate+L-Glutamate$$

$$Pyruvate+NADH \xrightarrow{LD} Lactate+NAD$$

The ALT assay was performed according to the manufacturers instructions. The ALT reagent was reconstituted in deionized water to contain L-alanine (400 μM), 2-oxoglutarate (12 μM), microbial LD (2000 U / L) and NADH (0.25 μM) at pH 7.4. For each sample, a 1 ml volume of the ALT reagent was placed in a cuvette and brought to 30 °C in a water bath. The test sample (100 μl) was then added and mixed with the ALT reagent by inversion. After 90 seconds at 30 °C, the absorbance of the mixture was read at 340 nm (Initial A). Continuing to incubate at 30 °C, the absorbance was read at after a further 30 seconds and 60 seconds (Final A). The rate of change in $O.D._{340}$ per min is calculated by subtracting Final A from Initial A. The ALT activity can be calculated using the millimolar absorbance of NADH at 340 nm which is 6.22 in the following equation.

$$ALT\ (U/L) = \frac{\Delta A \text{ per min} \times TV \times 1000}{6.22 \times LP \times SV}$$

where: ΔA per min = Change in absorbance per minute at 340 nm

TV = Total volume (1.1 ml)

SV = Sample volume (0.1 ml)

LP = Light path (1 cm)

1000 = Conversion of U / ml to U / L

6.2.7 Histological processing of liver biopsies.

After 24 hr formalin fixation, liver biopsies were carefully arranged in unicassettes (Raymond A Lamb, London, UK). Biopsies from individual tamarins were placed together in one cassette. The cassette lids were securely fitted and cassettes loaded into an automatic processor (Jung TP 1050, Leica UK Ltd, Milton Keynes, UK). The tissues were dehydrated by incubation for 4 hr in each of 70 %, 80 %, 90 %, 95 % and 100 % industrial methylated spirit (IMS) followed by a second 4 hr incubation in 100 % IMS. A 4 hr incubation in IMS / chloroform followed by 2 washes in chloroform (4 hr each) were performed in order to clear the tissues which were then impregnated with paraffin wax. The tissues were immersed in 3 changes of molten paraffin wax at 60 °C. The first incubation was for 4 hr followed by 2 further incubations for 2 hr each. The cassettes were then removed from the processor wax bath and transferred to a paraffin wax bath at 65 °C. Cassettes were individually removed from the paraffin wax bath and, working on a blockmaster stage heated to 60 °C, the biopsies were placed in a disposable mould filled with molten paraffin wax. The base of the cassette was placed onto the top of the mould which was then transferred to the cold plate of the blockmaster to cool at a temperature of -2 °C until the wax had set firmly. The blocks were then removed from the moulds.

The blocks were prepared for microtome cutting by trimming excess paraffin wax from the cassette. The cassette was then mounted onto the microtome and aligned parallel to the cutting edge of the knife. Sections were cut at a thickness of 10 μm and floated onto the surface of a water bath at 45 °C until fully stretched. The sections were then transferred to aminoproyltriethoxysilane (Sigma Chemical Co., St Louis, MO, USA) coated slides by submerging behind the sections and drawing the slides up so that the sections became attached. Two sections were placed on each slide. The slides were placed in an incubator at 45 °C until dry.

Prior to use, sections were dewaxed by submersion in 2 changes of xylene for 5 min each followed by 2 washes in absolute alcohol for 5 min each. The section were then air dried before rehydration in 70 % alcohol for 4 min and 2 changes of distilled water for 4 min each.

6.2.7.1 Haematoxylin and eosin staining

Rehydrated sections were submerged in Harris' haematoxylin stain (BDH) for 10 seconds and washed in a running tap water bath for 5 min. Differentiation was achieved by incubating the sections in 1 % HCl, 70 % alcohol for 5 seconds and again washing in tap water as above. Sections were then stained in 1% eosin (yellowish) for 10 min. After a 5 minute wash in running tap water, sections were dehydrated by 2 minute incubations in each of 50 %, 70%, 90 %, 95 % and 100 % alcohol followed by clearing in xylol for 2 min and mounting in DPX. Stained sections were viewed by light microscopy and post inoculation biopsies were compared with those taken prior to inoculation.

6.2.7.2 Immunohistological detection of HAV

Dewaxed sections were placed in 0.05 % hydrogen peroxide in methanol for 10 min to inactivate endogenous phosphatase activity followed by a 5 min PBS wash . Slides were then placed in a 1 l beaker containing 250 ml sodium citrate buffer which was covered and microwaved at 850 W (Panasonic) for 40 min in order to increase the permeability of the cells. After a 5 min wash in PBSA, sections were overlaid with 50 µl of IgG affinity purified human post-convalescent serum diluted 1:250 and PBSA and incubated in a moist chamber at room temperature for 4 hr. Sections were then washed in 3 changes of PBSA for 15 min each before incubation with anti-human IgG - alkaline phosphatase conjugate (Sigma Chemical Co.) diluted 1:500 in PBSA for 1 hr. After washing in PBSA as previously, bound antibody wash visualised by incubation of sections with bromochloroindolylphosphate / nitro blue tetrazolamine (BCIP / NBT stable preparation, Life Technologies Inc.) until sufficient colour was visible by light microscopy, usually approximately 1 hr. Slides were rinsed in tap water and counter stained with Harris' haematoxylin stain (BDH) for 10 seconds. Stained sections were rinsed, dehydrated and mounted in DPX as above. All incubations were performed at room temperature.

As a negative control, a section from each liver biopsy was treated as above except for being incubated with human serum which had previously been found to be negative for anti-HAV rather than primary affinity purified IgG from human post-convalescent serum. No immunostaining was seen in these sections.

6.3 RESULTS

6.3.1 Detection of HAV RNA in faecal samples

Prior to inoculation with HAV, all faecal samples were tested by RT-PCR and found to be negative for HAV RNA. In 2 out of the 3 Group 1 tamarins, inoculated with the wild type HAV HM175, viral RNA was detected as early as 4 days p. i. (Table 6.3, Figures 6.2 and 6.3). Faecal shedding of HAV commenced between days 4 and 9 in the third Group 1 tamarin. Maximum titres of up to 11 $\log_{10}$ genomes g^{-1} of faeces (tamarin C9, 14 days p.i.) were detected in this group between days 14 and 21 p. i.. The duration of excretion of the wild type virus as detected by reverse transcription and PCR varied from 31 to 54 days.

In each of the tamarins infected with HAV HM175/18f (Group 2), faecal shedding occurred 4 days after inoculation (Table 6.3, Figure 6.4). Excretion of viral RNA reached a peak at 9 days p.i.. However, maximum titre detected in this group was only 7.5 $\log_{10}$ genomes g^{-1} of faeces (Tamarin C13, 9 days p.i.) and the duration of faecal shedding was between 10 and 17 days. No HAV RNA was detected in Group 3 tamarins infected with the cytopathic HAV HM175A.2

6.3.2 HAV Viraemia

HAV RNA was only detected by quantitative RT-PCR in Group 1 tamarins which were inoculated with the wild type HM175 (Table 6.4). In tamarins C16 and C20, only one serum sample was viraemic at 14 and 21 days respectively whereas in tamarin C9, viraemia was detected from day 9 to day 17 p.i.. The maximum titre of HAV RNA detected in the serum samples was 4.3 $\log_{10}$ genomes ml^{-1} (tamarin C9, 17 days p.i.).

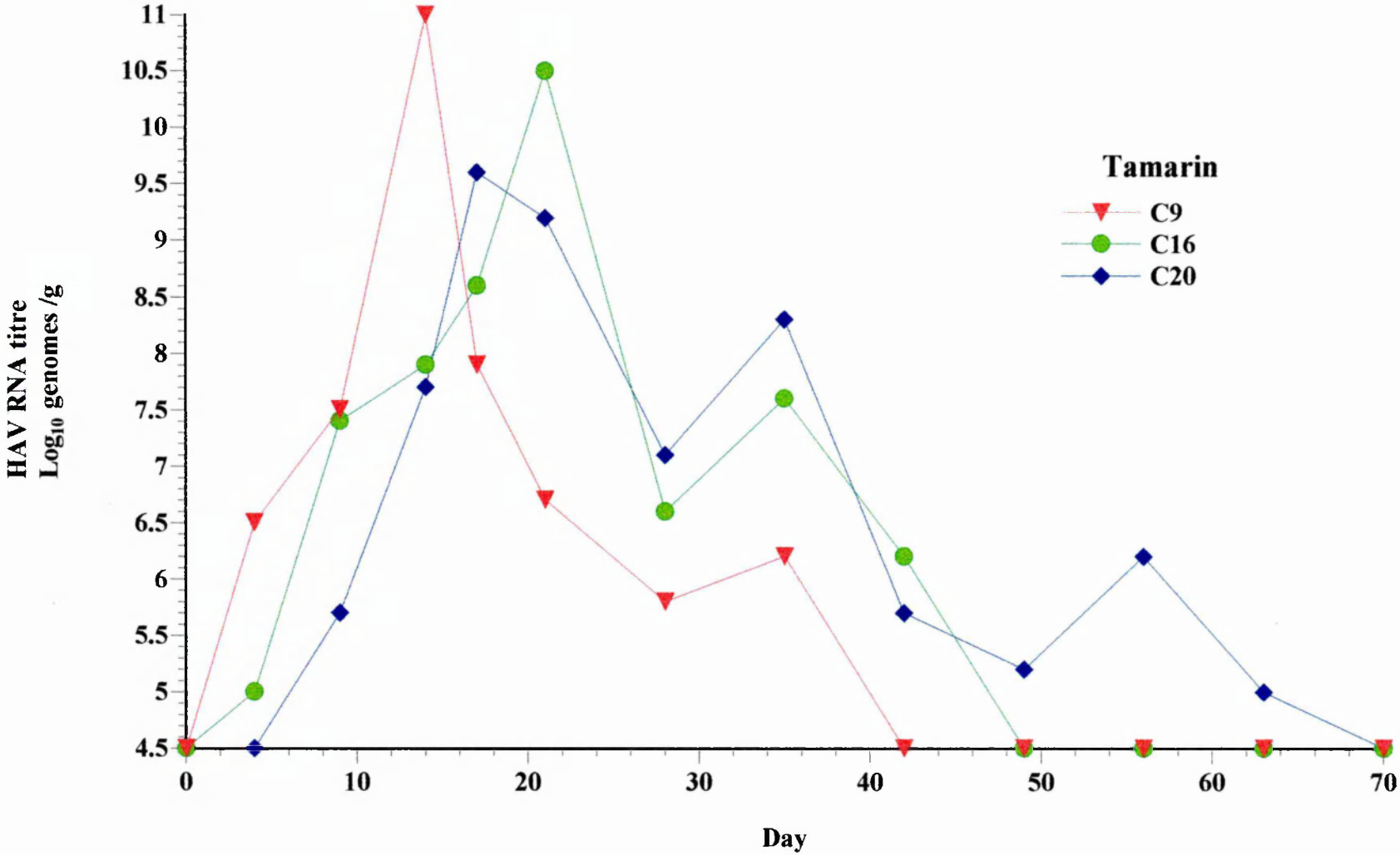
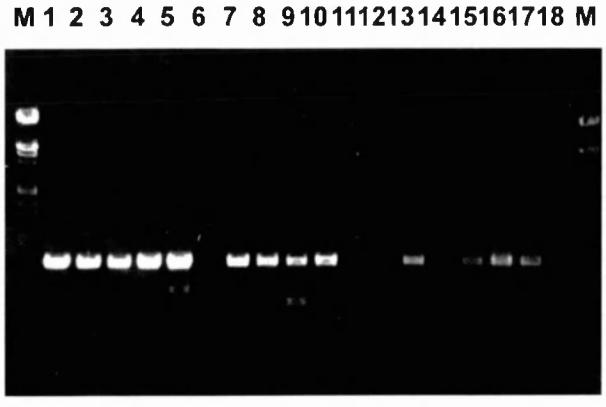
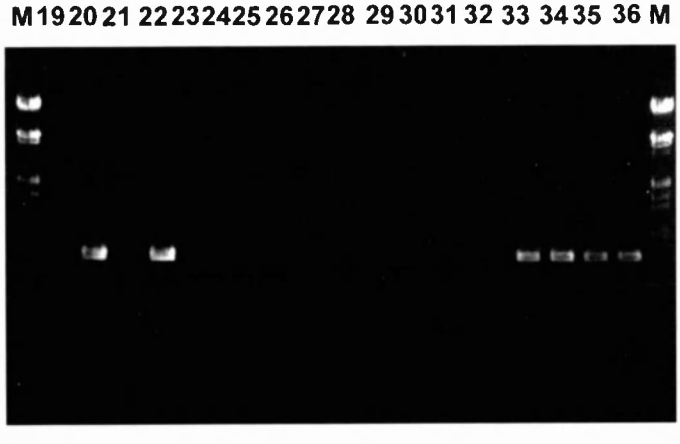


Figure 6.2 Faecal shedding of HAV HM175/wt

Figure 6.3 Example quantitative RT-PCR assay of HAV RNA in tamarin faeces





Samples (Faecal sample from tamarin C9, 9 days post inoculation)

M	molecular weight markers 1		
1-5	cDNA diluted $1:10^{2}$	19-23	cDNA diluted $1:10^{3.5}$
6	negative control water	24	Negative control water
7-11	cDNA diluted $1:10^{2.5}$	25-29	cDNA diluted $1:10^{4}$
12	negative control water	30-32	Negative control water
13-17	cDNA diluted $1:10^{3}$	33+34	Standard CS101 1:10
18	negative control water	35+36	Standard CS101 1:100

Calculation of HAV RNA titre

Result of GLIM analysis = 883 genomes / sample

log titre = $\log (88 \times (100/8.8)^{a} \times (1000/3.5)^{b} \times 10^{c})$

= 7.5 log genomes g^{-1}

[a] Replication efficiency factor
[b] Volume factor (1ml)
[c] Faecal suspension = 0.1 g / ml

Table 6.3. Faecal excretion of HAV RNA

Day	Virus titre (log_{10} genomes g^{-1}) Group 1 HM175/wt			Virus titre (log_{10} genomes g^{-1}) Group 2 HM175/18f			Virus titre (log_{10} genomes g^{-1}) Group 3 HM175A.2		
	C9	C16	C20	C13	C17	C19	C12	C15	D3
0	0	0	0	0	0	0	0	0	0
4	6.5	5	0	5	5	5	0	0	0
9	7.5	7.4	5.7	7.5	7.4	5.6	0	0	0
14	11	7.9	7.7	6.6	5.6	5.5	0	0	0
17	7.9	8.6	9.6	0	6	5.6	0	0	0
21	6.7	10.5	9.2	0	6.8	0	0	0	0
28	5.8	6.6	7.1	0	0	0	0	0	0
35	6.2	7.6	8.3	0	0	0	0	0	0
42	0	6.2	5.7	0	0	0	0	0	0
49	0	0	5.2	0	0	0	0	0	0
56	0	0	6.2	0	0	0	0	0	0
63	0	0	5	0	0	0	0	0	0
70	0	0	0	0	0	0	0	0	0

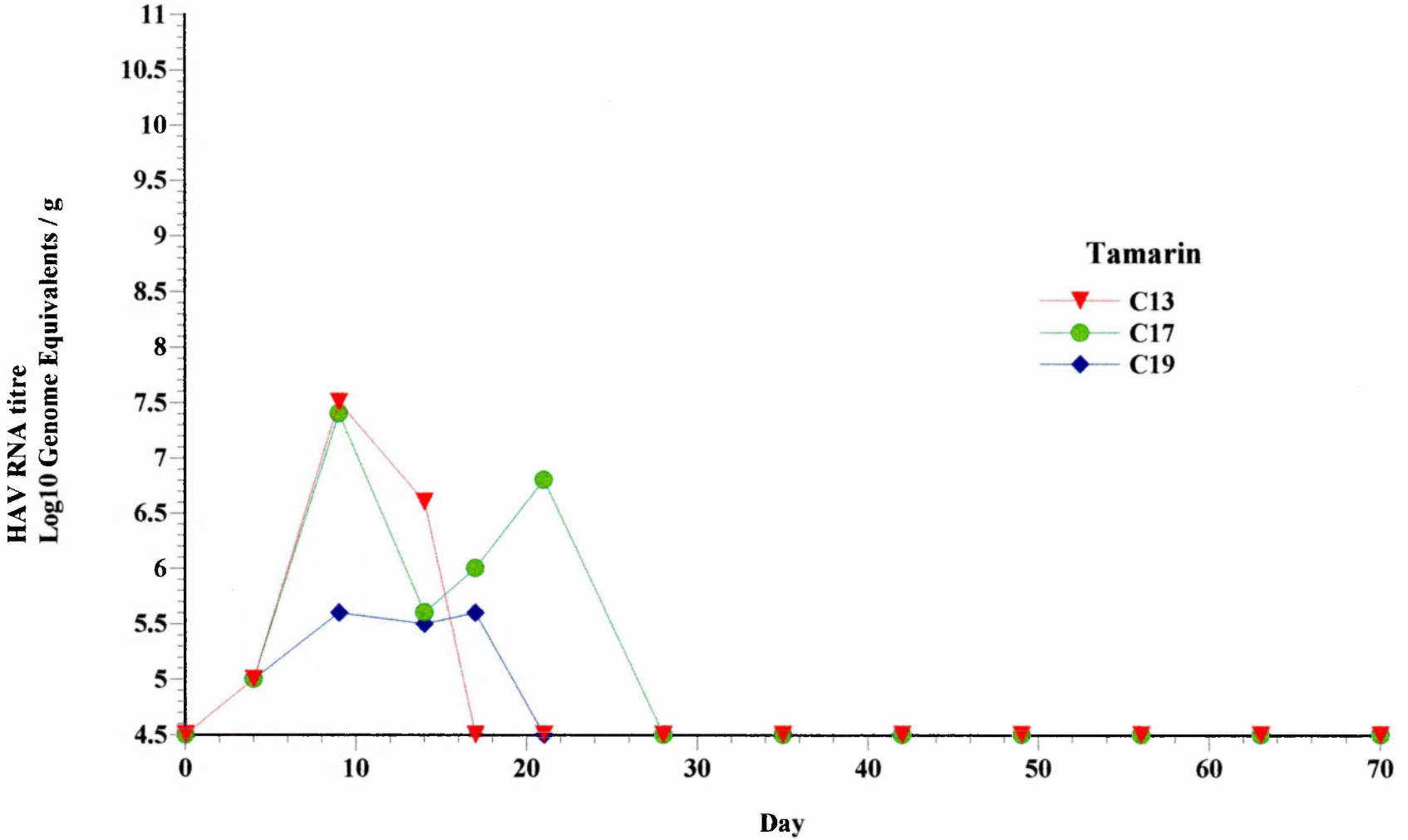


Figure 6.4 Faecal shedding of HAV HM175/18f

Table 6.4 HAV viraemia in Group 1 tamarins (HM175/wt)

Day	Virus titre genomes ml^{-1}		
	C9	C16	C20
0	0	0	0
4	0	0	0
9	4.1	0	0
14	3.3	3.2	0
17	4.3	0	0
21	0	0	3.8
28	0	0	0
35	0	0	0
42	0	0	0
49	0	0	0
56	0	0	0
63	0	0	0
70	0	0	0

6.3.3 Cytopathic microtitre plate assay of HAV HM175A.2

Faecal and serum samples from Group 3 animals inoculated with HAV HM175A.2, the cytopathic variant, were analysed by cytopathic microtitre plate assay. No virus was detected in any of these samples.

6.3.4 Immunological response

The production of antibodies was monitored by enzyme immunoassay of serum samples. Two kits were used to test the samples and the performance of the positive and negative controls and calculated cut off values are shown in Table 6.5. Test validity values of above 0.3 indicate that both of the assays were successful. The calculated cut off values were 0.428 and 0.419. Readings lower than these values indicate that the sample is positive. The results of enzyme immunoassay of the tamarin sera are shown in Table 6.6. Antibodies to HAV were detected in all tamarins regardless of the HAV variant inoculated. Seroconversion occurred between days 14 and 28 p.i.. All tamarin serum samples then remained positive for total anti-HAV to the end of the time course.

Table 6.5. Performance of Murex HAV VK38 assays

		Assay 1	Assay 2
Negative control	1	0.732	0.765
	2	0.849	0.794
	3	0.792	0.776
Mean negative control (N)		0.791	0.778
Positive control	1	0.066	0.060
	2	0.065	0.059
Mean positive control (P)		0.066	0.06
Test validity value (N-P)		0.725	0.719
Cut off value (N+P)/2		0.428	0.419

6.3.5 Circulating ALT levels

All tamarin serum samples were tested for changes in ALT levels. A cut-off value for normal ALT levels was calculated as the mean of 30 serum samples taken before inoculation on days -13 and 0 (Table 6.7), plus 3 times the standard deviation. The 30 normal serum samples included 12 pre-inoculation samples taken from groups of tamarins not otherwise used in this study. The mean was 5 U L^{-1} with a standard deviation of 3, therefore the normal cut-off was calculated to be 14 U L^{-1}. Serum levels of ALT in the Group 1 tamarins are shown in Figure 6.5. In 2 of the tamarins (C16 and C20) the ALT levels rose above the cut-off level by day 9 p.i. and tamarin C9 showed abnormal ALT levels by day 14 p.i.. A peak of 69 ALT U L^{-1} was seen on day 14 in tamarin C20 however, the other Group 1 tamarin serum levels were not at a maximum until 21 days p.i.. The highest titre of serum ALT found in Group 1 animals was 90 ALT U L^{-1} (6 x the cut-off value). Serum ALT levels in Group 1 animals returned to normal between 35 and 42 days p.i..

Table 6.6. Detection of total anti-HAV in inoculated tamarins

Day	$O.D._{450}$ Group 1 HM175/wt			$O.D._{450}$ Group 2 HM175/18f			$O.D._{450}$ Group 3 HM175A.2		
	C9	C16	C20	C13	C17	C19	C12	C15	D3
0	0.734	0.708	0.706	0.689	0.758	0.822	0.952	0.733	0.799
4	0.809	0.712	0.764	0.650	0.658	0.679	0.891	0.917	0.871
9	0.758	0.739	0.778	0.686	0.732	0.792	0.754	0.872	0.925
14	0.769	0.662	0.384	0.368	0.763	0.727	0.648	0.843	0.705
17	0.602	0.703	0.115	0.136	0.320	0.301	0.154	0.819	0.228
21	0.366	0.617	0.059	0.080	0.092	0.105	0.097	0.729	0.119
28	0.070	0.379	0.059	0.071	0.072	0.068	0.080	0.110	0.075
35	0.056	0.068	0.051	0.062	0.067	0.064	0.073	0.079	0.072
42	0.053	0.052	0.048	0.072	0.082	0.063	0.074	0.086	0.068
49	0.063	0.061	0.053	0.064	0.069	0.064	0.066	0.084	0.066
56	0.054	0.056	0.060	0.067	0.070	0.059	0.063	0.083	0.072
63	0.054	0.074	0.065	0.061	0.064	0.072	0.057	0.072	0.064
70	0.057	0.057	0. 061	0.068	0.065	0.063	0.060	0.087	0.066

Table 6.7 Levels of serum ALT in HAV inoculated tamarins

Day	Serum ALT (U L^{-1}) Group 1 HM175/wt			Serum ALT (U L^{-1}) Group 2 HM175/18f			Serum ALT (U L^{-1}) Group 3 HM175A.2		
	C9	C16	C20	C13	C17	C19	C12	C15	D3
-13	4	5	2	7	11	9	2	5	7
0	2	9	4	2	9	2	7	11	2
4	2	9	5	11	5	9	4	2	0
9	11	30	16	14	16	19	11	7	2
14	16	11	69	12	16	27	4	2	4
17	27	23	28	11	12	14	4	4	2
21	60	90	9	5	46	28	7	2	5
28	16	16	25	4	11	14	9	4	0
35	21	12	19	4	21	5	5	2	7
42	9	4	5	5	11	18	7	2	0
49	14	4	7	2	25	4	4	5	11
56	4	2	4	4	2	9	2	2	12
63	7	5	4	0	2	7	4	7	4
70	0	7	5	5	4	2	2	9	4

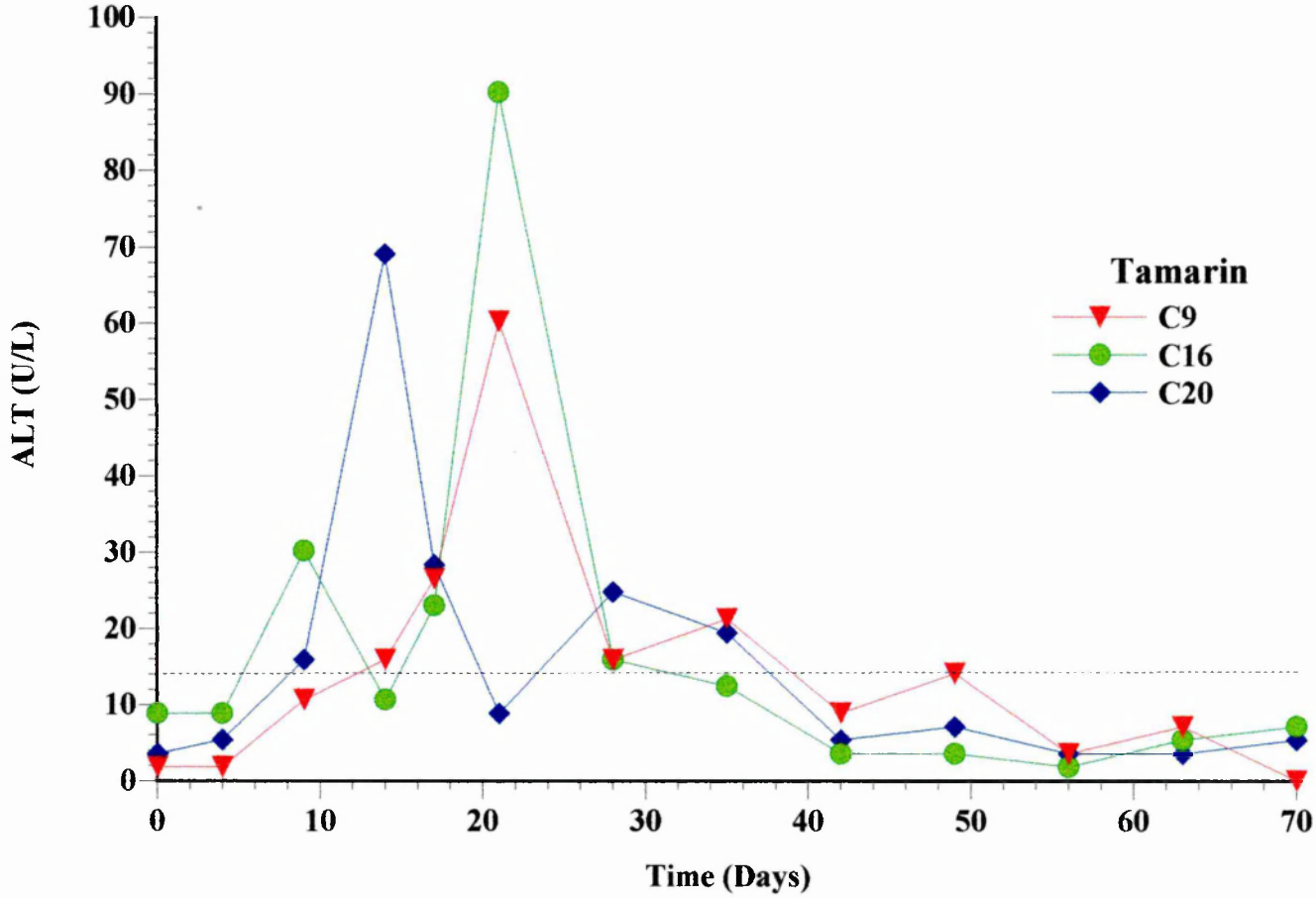


Figure 6.5. Serum ALT levels in Group 1 tamarins (HAV HM175/wt)

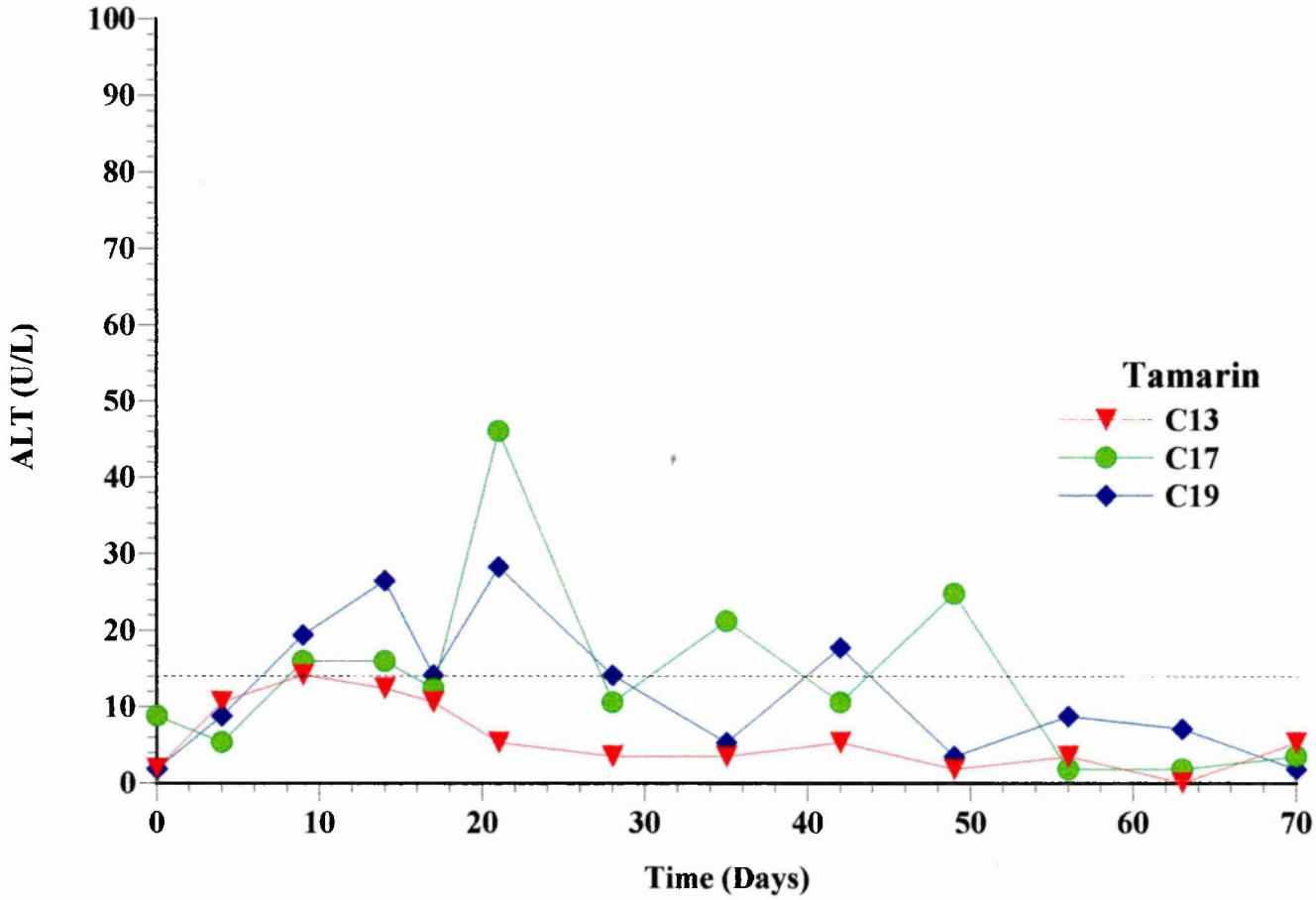


Figure 6.6. Serum ALT levels in Group 2 tamarins (HM175/18f)

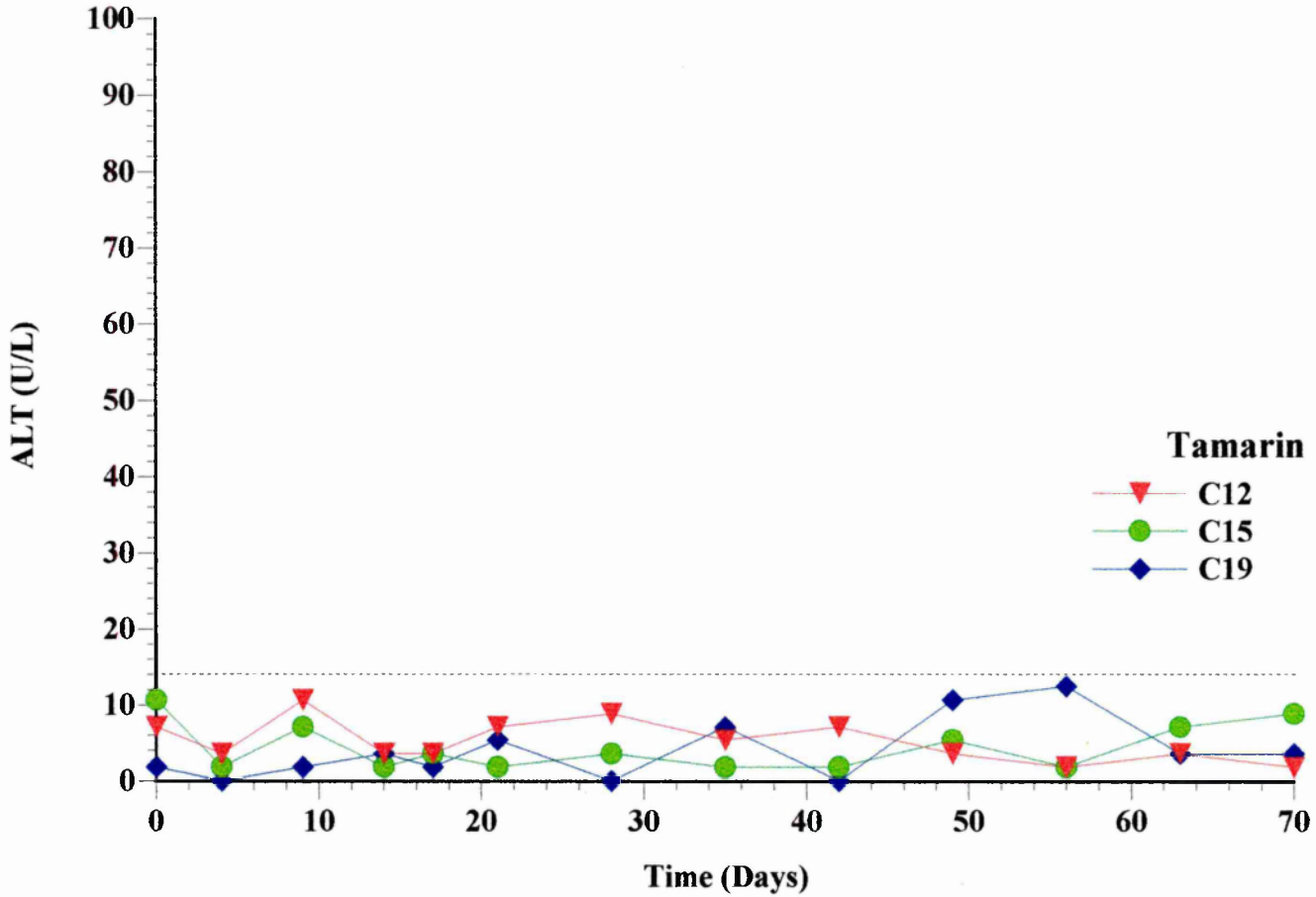


Figure 6.7. Serum ALT levels in Group 3 tamarins (HM175A.2)

Only 2 of the tamarins (C17 and C19) inoculated with HAV HM175/18f had markedly elevated ALT levels (Figure 6.6). Peak ALT titres were detected in these animals at 21 days p.i. and the highest titre found in Group 2 was 46 U L^{-1} (3 x the cut-off value). In tamarin C13, the serum ALT levels at 9 to 14 days p.i. were at the cut-off value. The ALT levels returned to normal in tamarins C17 and C19 at days 56 and 49 p.i. respectively. Serum ALT levels in all of the Group 3 animals inoculated with HAV HM175A.2 remained normal throughout the timecourse (Figure 6.7).

6.3.6 Liver histopathology

Liver biopsies were taken from each animal 13 days prior to inoculation, 21 days and 70 days p.i.. Light microscopy of haematoxylin and eosin stained sections of all biopsies taken prior to inoculation showed normal liver histology (Figure 6.8, Table 6.8). At 21 days p.i., all biopsies taken from the tamarins in Group 1, infected with the wild type virus, showed signs of liver damage characteristic of viral infection. Lymphocytic and macrophage infiltration was mainly seen around the portal tracts and the lobular periphery. Due to the small size of the biopsies, it was difficult to determine whether infiltrating cells connected neighbouring portal tracts. To a lesser extent, cellular infiltration was also seen around central hepatic veins. In the parenchyma, focal areas of infiltration and necrosis were demonstrated. Pathological changes were visible in sections of the 70 day biopsies, however, at this stage, the infiltration had receded and was generally only seen around the portal tracts.

Only one of the biopsies taken from the tamarins infected with HAV HM175/18f at 21 days showed signs of liver damage characterised by infiltration and enlargement of portal tracts. Occasional areas of infiltration and necrosis was also seen in the parenchyma to a lesser extent than in the biopsies of group 1 tamarins. Two large areas of periportal

Figure 6.8 Histological analysis of tamarin liver biopsies

Group 1 (HAV HM175/wt)

a) Pre-inoculation biopsy

h- hepatocytes

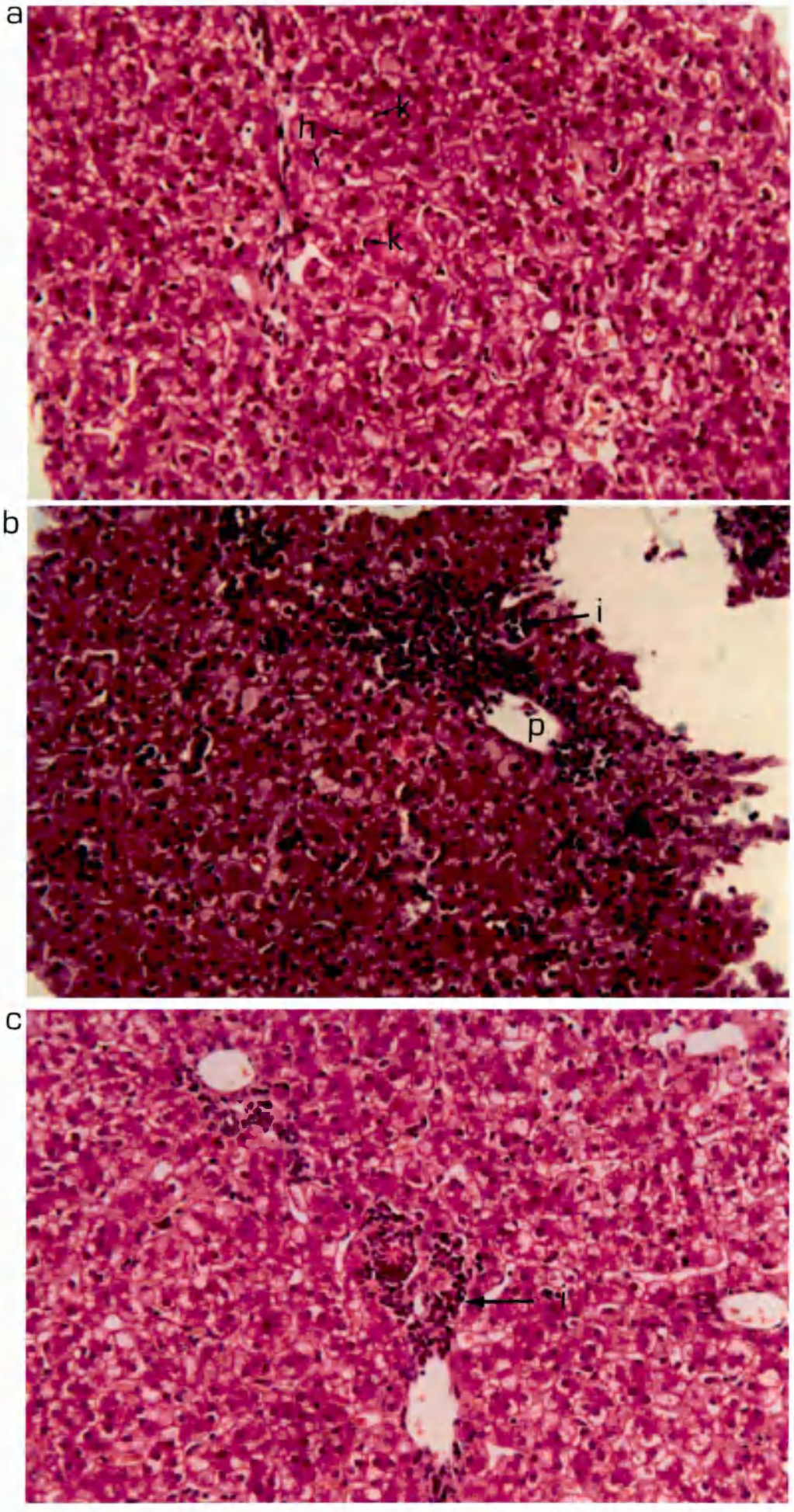
k- Kupffer cells

b) 21 day post inoculation biopsy

i- cellular infiltration

p- hepatic portal vein

c) 70 day post inoculation biopsy


a
h
k
k
b
i
p
c
i

(Figure 6.8 continued)

Group 2 (HAV HM175/18f)

d) Pre-inoculation biopsy

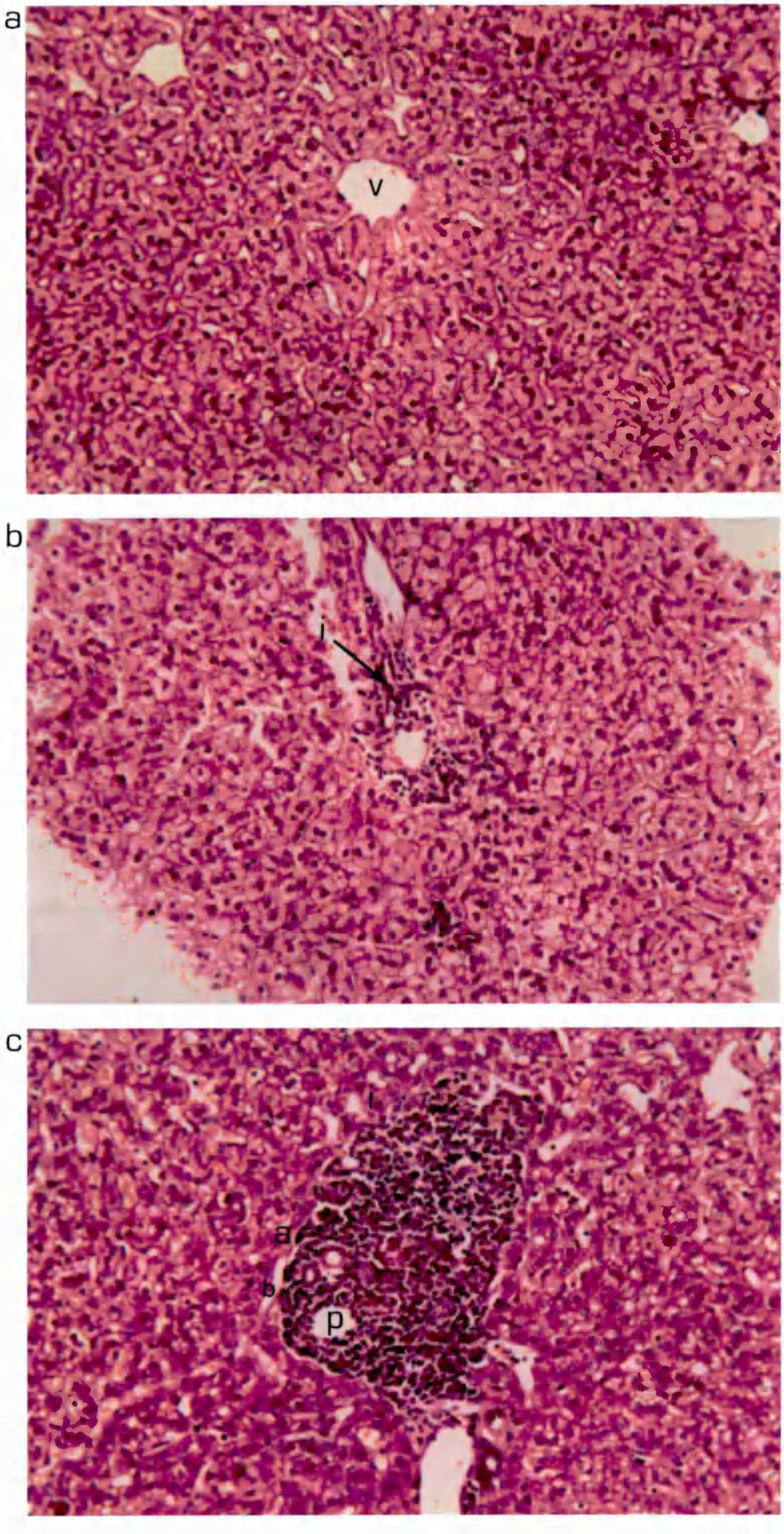
v- central vein

e) 21 day post inoculation biopsy

i- cellular infiltration

f) 70 day post inoculation biopsy

a- hepatic artery
b- bile duct
p- hepatic portal vein
} portal tract


a
v
b
i
c
l
a
b
p

(Figure 6.8 continued)

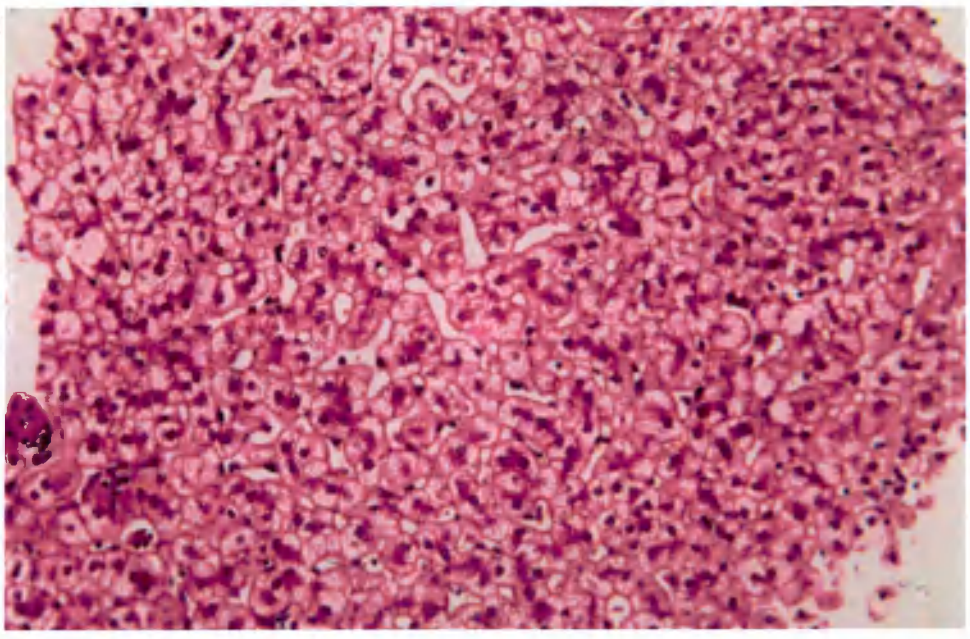
Group 3 (HAV HM175A.2)

g) Pre-inoculation biopsy

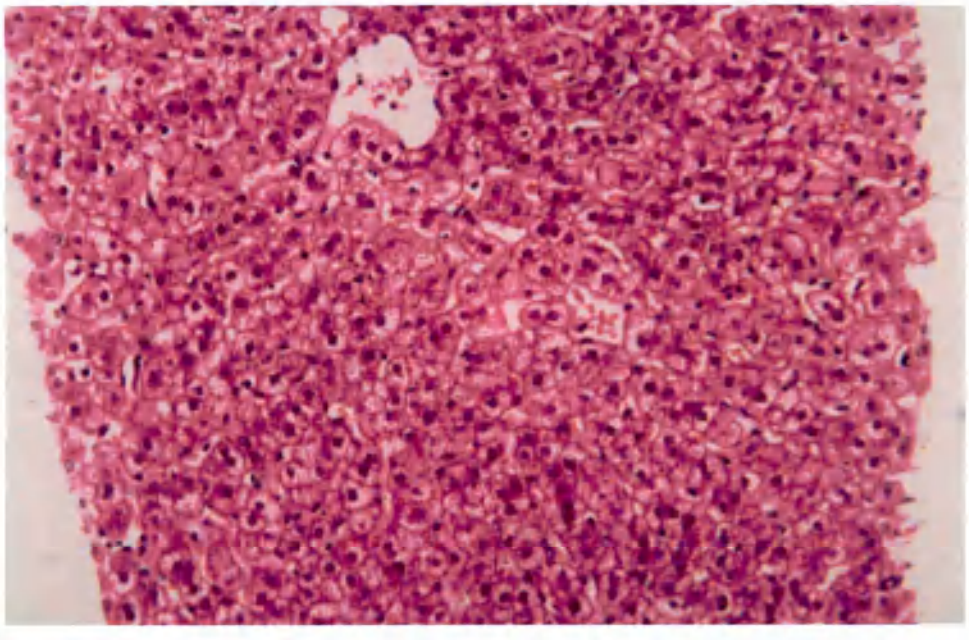
h) 21 day post inoculation biopsy

i) 70 day post inoculation biopsy

a



b



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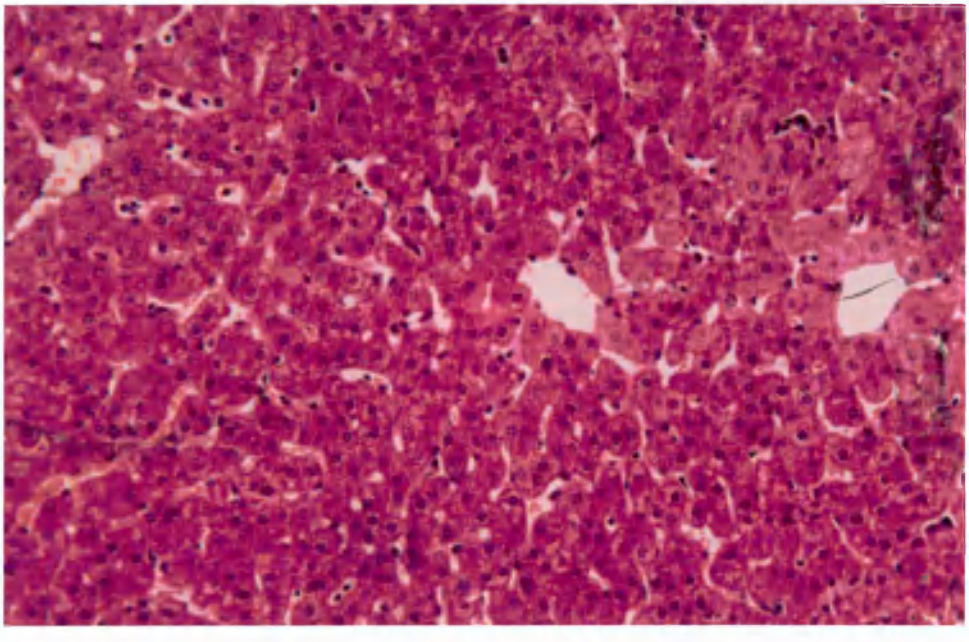


Table 6.8 histological analysis of liver biopsies from HAV infected tamarins

	Group 1 (HM175 wild type)			Group 2 (HM175/18f)			Group 3 (HM175A.2)		
Biopsy	**C9**	**C16**	**C20**	**C13**	**C17**	**C19**	**C12**	**C15**	**D3**
-13 day	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
21 day pi	Small areas of lymphocyte infiltration	Lymphocytic infiltration /hepatocyte degeneration	Lymphocytic infiltration /hepatocyte degeneration	Normal	+/- infiltrates	Normal	Normal	Normal	Normal
70 day pi	Small areas of lymphocyte infiltration	Small areas of lymphocyte infiltration	Small areas of lymphocyte infiltration	ND	1 area of infiltration	Normal	Normal	Normal	Normal

infiltration were seen in the 70 day liver biopsy from the same tamarin and no damage was visible in biopsies from the other group 2 tamarins.

None of the liver biopsies from the Group 3 tamarins, inoculated with HM175A.2, showed any signs of liver pathology.

6.3.7 Immunohistological staining of HAV in liver biopsy sections

Immunohistology was performed on sections from each of the tamarin biopsies using human post-convalescent serum and alkaline phosphatase conjugated anti-human IgG. Very little background staining was seen in sections taken from biopsies of 13 days prior to inoculation (Figure 6.9, Table 6.9). The pale pink patches in the centre of each photomicrograph is an artefact of photography. HAV antigen was demonstrated in 2 of the Group 1 tamarins at 21 days p.i. however, no positive signal was detected in the corresponding biopsy of tamarin C20. In the positive sections, staining was seen in the cytoplasm of hepatocytes and Kuppfer cells. The distribution of HAV antigen appeared to be confined to individual cells and in portal tracts and parenchyma with a random distribution. At 70 days post infection, none of the Group 1 tamarin biopsies were positive for HAV antigen.

Very low levels of viral antigen were demonstrated as dense staining in the cytoplasm of occasional hepatocytes in only one of the tamarins (C17) inoculated with HAV HM175/18f at 21 days p.i.. Light, granular HAV antigen staining was detected in the cytoplasm of cells around a single large area of infiltration seen in the 70 day p.i. biopsy of tamarin C17. All biopsies from the remaining 2 tamarins in Group 2 and each of the Group 3 animals were negative for HAV antigen.

Figure 6.9 Immunohistochemical staining of HAV antigen in tamarin liver biopsies

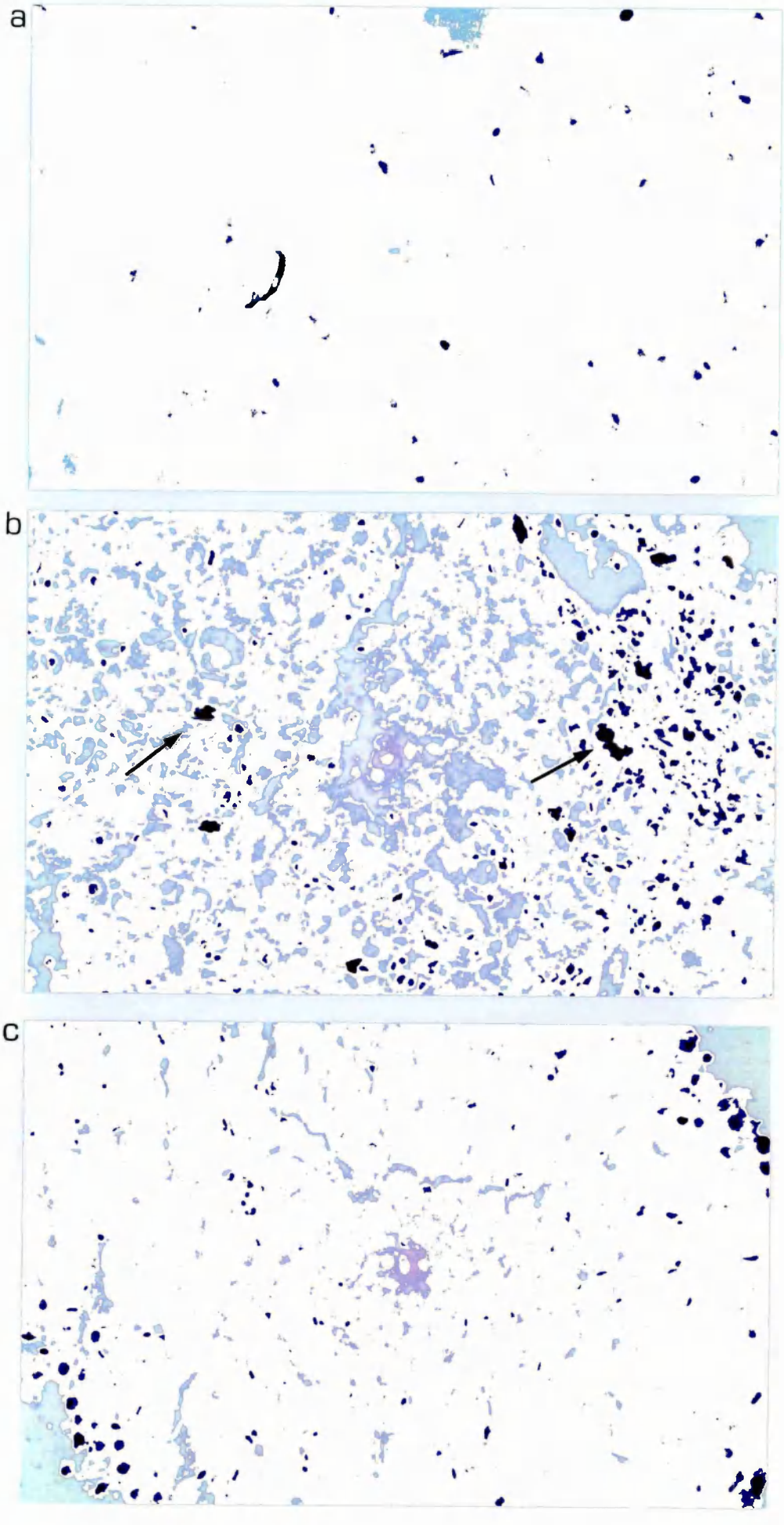
⟶ Immunohistochemically stained HAV antigen

Group 1 (HAV HM175/wt)

a) Pre-inoculation biopsy

b) 21 day post inoculation biopsy

c) 70 day post inoculation biopsy


a
b
c

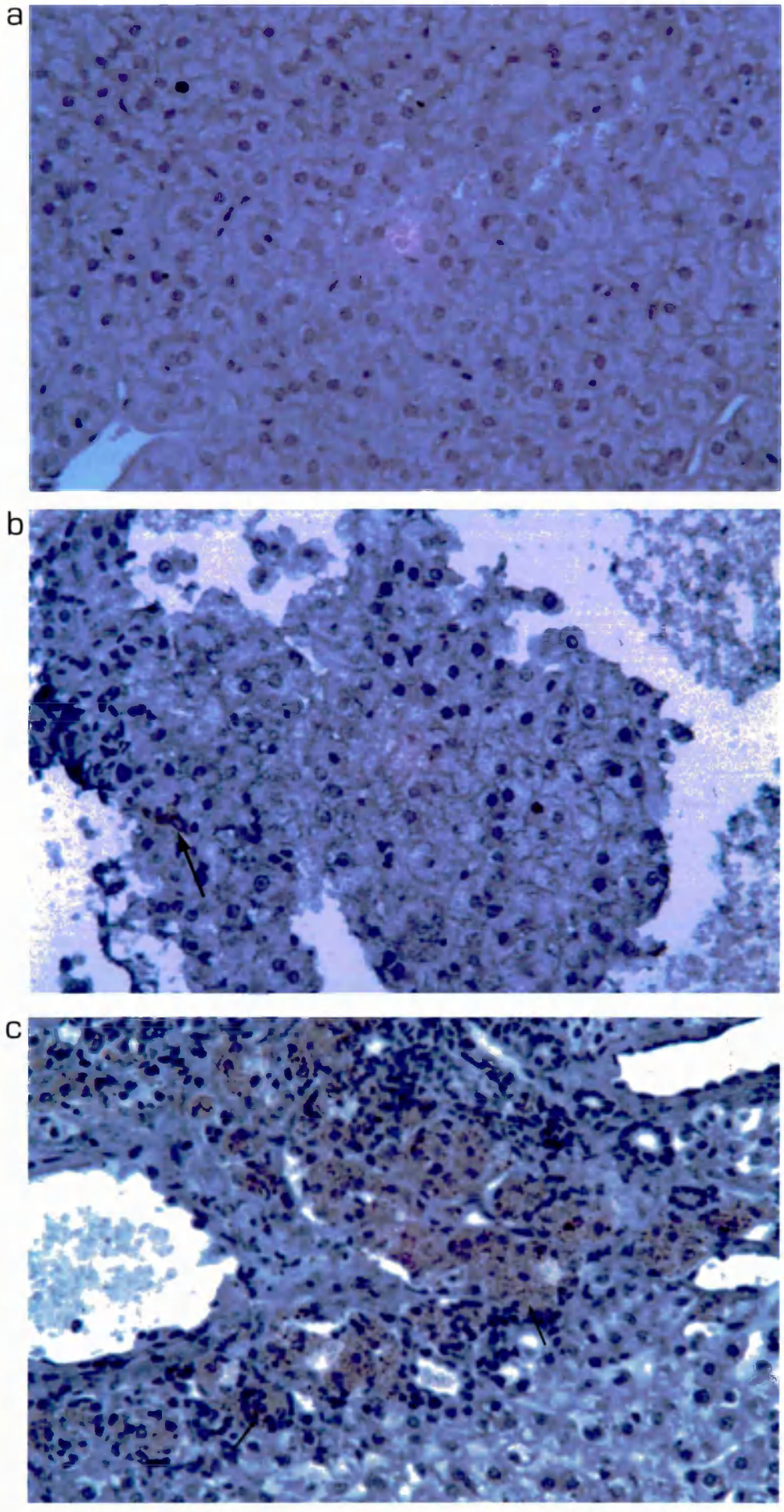
(Figure 6.9 continued)

Group 2 (HAV HM175/18f)

d) Pre-inoculation biopsy

e) 21 day post inoculation biopsy

f) 70 day post inoculation biopsy


a
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(Figure 6.9 continued)

Group 3 (HAV HM175A.2)

g) Pre-inoculation biopsy

h) 21 day post inoculation biopsy

i) 70 day post inoculation biopsy

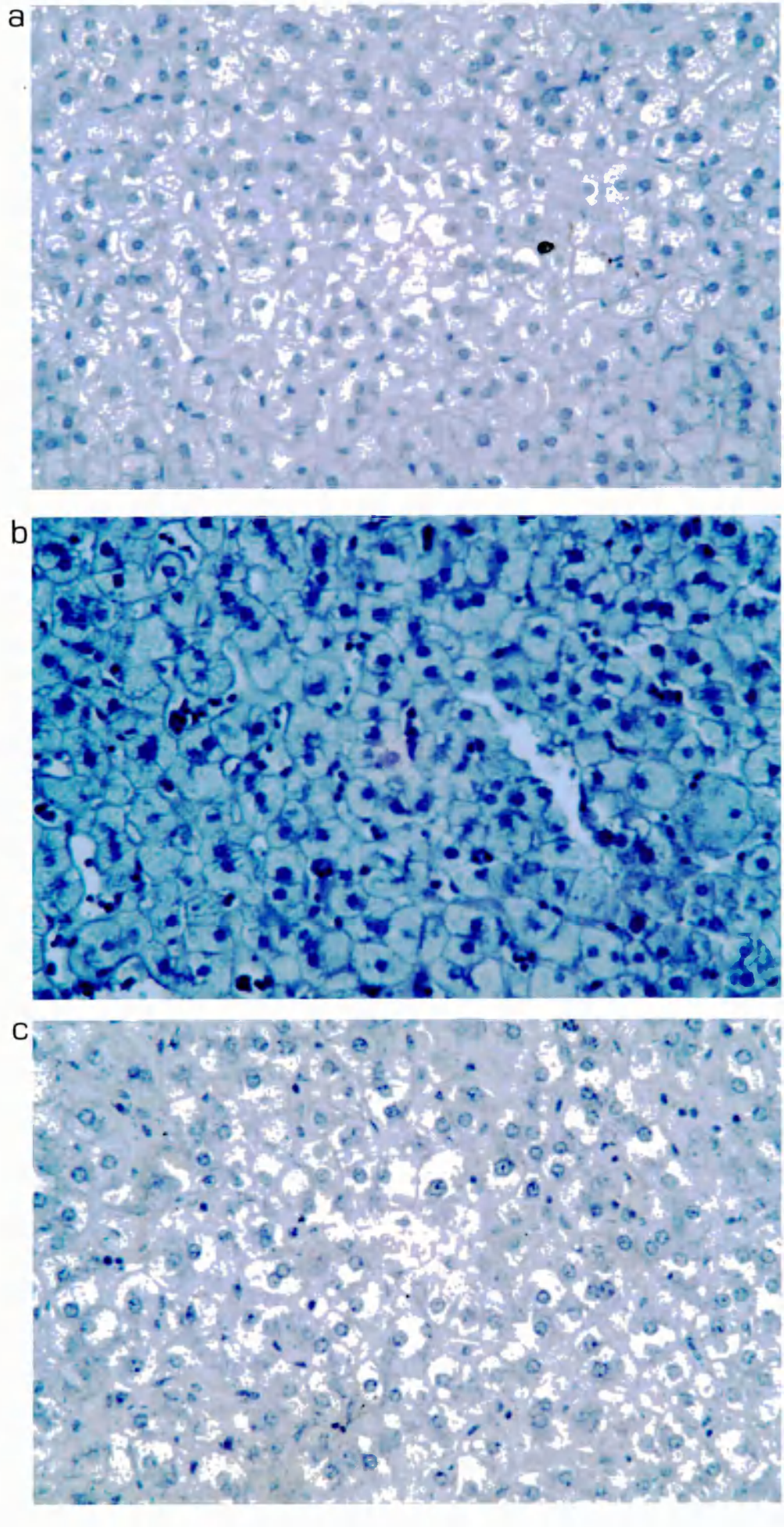

a
b
c

Table 6.9 Immunohistochemical analysis of liver biopsies from HAV infected tamarins

	Group 1 (HM175 wild type)			Group 2 (HM175/18f)			Group 3 (HM175A.2)		
Biopsy	**C9**	**C16**	**C20**	**C13**	**C17**	**C19**	**C12**	**C15**	**D3**
-13 day	-	-	-	-	-	-	-	-	-
21 day pi	+	++	-[a]	-	+	-	-	-	-
70 day pi	-	-	-	ND	+[b]	-	-	-	-

[a] very small biopsy taken
[b] Staining around area of infiltration only

6.4 DISCUSSION

The virulence of two tissue culture adapted strains of HAV, HM175/18f and HM175A.2 were compared in a tamarin model. Intravenous inoculation of the three virus preparations was performed as this method appears to be more sensitive than oral inoculation in comparing virulence (Purcell *et al.*, 1984).

The replication of HAV in the tamarin model was detected and a quantitative measure of viral shedding in the faeces obtained by RT-PCR. This technique was useful for the direct comparison of the growth wild type and tissue culture adapted variants of HAV HM175 in tamarins. The results show that the wild type virus replicated well, producing up to 10^{11} HAV genome equivalents per gram of faeces. Faecal shedding was detected as early as 4 days after inoculation of the tamarins with wild type HAV and continued for up to 54 days by RT-PCR. Using a molecular hybridisation technique to detect HAV RNA in marmoset faeces after inoculation with 2×10^6 CID_{50} of wild type HAV HM175, (Ticehurst *et al.*, 1987) levels of up to 10^{10} genomes ml^{-1} were detected. The excretion of HAV RNA reported by Ticehurst *et al.* was comparable to those obtained in the present study by quantitative PCR throughout the time course with faecal shedding commencing at 4 days post inoculation and peak titres at 10-12 days post inoculation followed a slow decline in shedding. Inoculation of marmosets with a lower dose (10^3 CID_{50}) of the wild type HM175 (Karron *at al.*, 1988) resulted in a shorter duration of faecal shedding as detected by cDNA hybridisation of HAV RNA (2 weeks) and lower titres (maximum, 8.2 log genomes ml^{-1}).

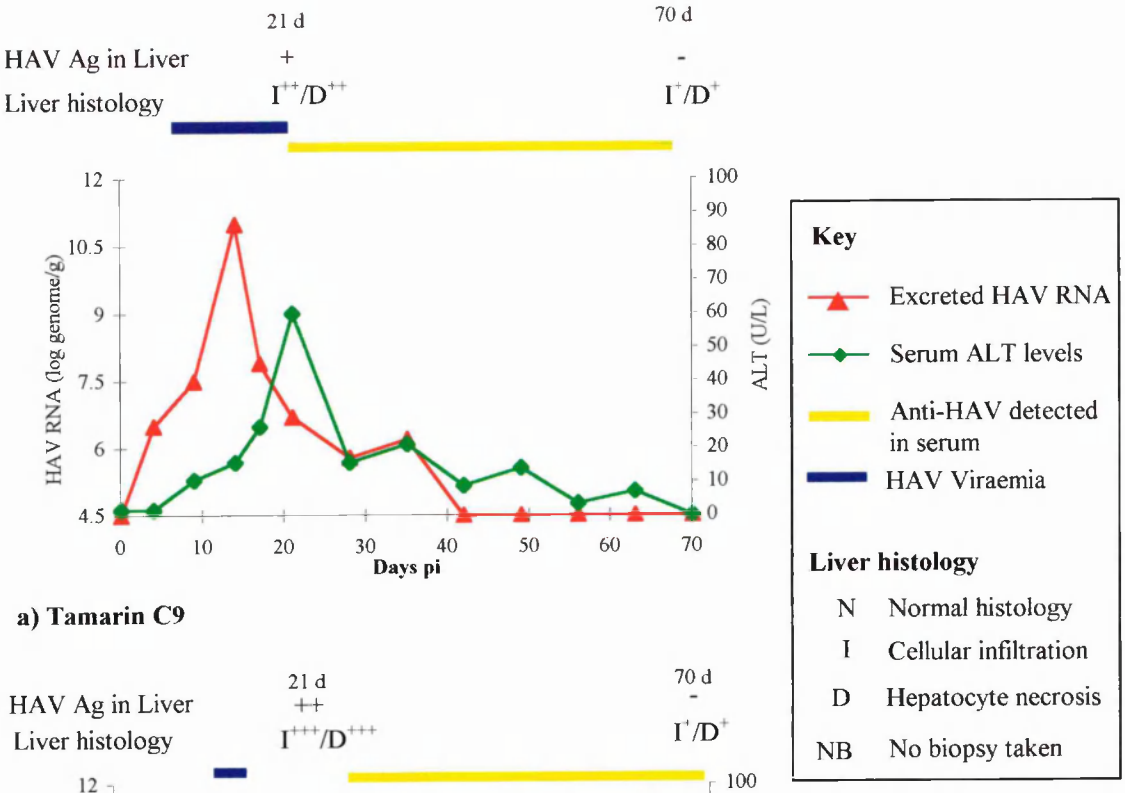
Initially, replication of the tissue culture adapted variant, HM175/18f, was similar to that of the wild type with an onset of faecal shedding in all animals detected 4 days after inoculation. Levels of excreted HAV RNA at 9 days post inoculation were also similar in the two groups with titres of 5.7 - 7.5 log_{10} genomes g^{-1} in animals inoculated with the wild

type virus and 5.6 - 7.5 $\log_{10}$ genomes g^{-1} with HM175/18f. Subsequently however, faecal shedding decreased with the tissue culture adapted strain while a further increase in viral titre was seen with the wild type.

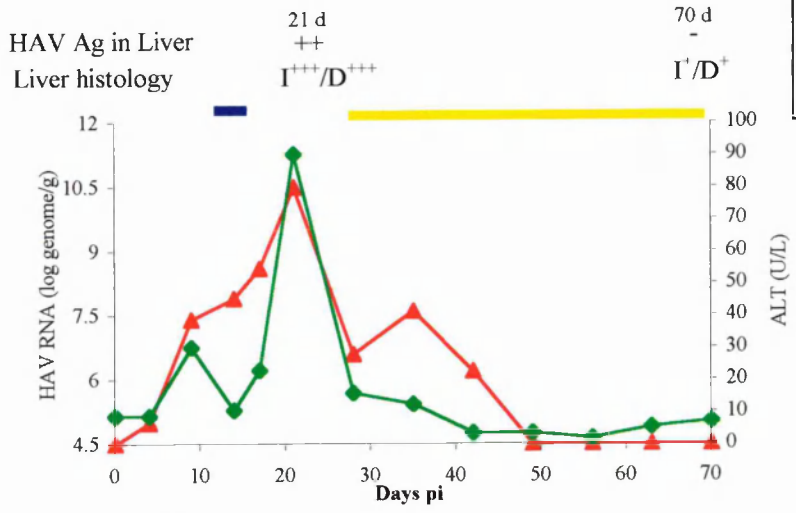
Lemon *et al.* (1991) showed that upon adaptation to growth in cell culture, antigenic variation may spontaneously arise in the absence of selective antibody pressure. The rapid decline in excretion of HM175/18f occurred at the time when anti-HAV was first detected in the tamarin sera, whereas in tamarin C20, inoculated with HM175/wt, the titre of virus in stool samples continued to increase in the presence of circulating antibodies to HAV. An alteration in antigenicity may have increased the susceptibility of the HM175/18f variant to neutralisation and resulted in more efficient clearance from the tamarin liver. While HAV HM175/18f is of the single wild type serotype, several mutations have been found in the proposed P1 region of the genome, which encodes the structural proteins, resulting in 3 amino acid changes (Table 6.10, Lemon *et al.*, 1991). These changes do not appear in the immunodominant anitgenic site, which involves the residues Ser-102, Val-171 and Ala-176 of VP1 as well as Asp-70 and Gln-74 of VP3 (Ping and Lemon, 1992). However, they may alter the capsid conformation such that the affinity of neutralising antibodies is increased resulting in more efficient neutralisation.

Table 6.10 Amino acid alterations in the P1 region of HM175 p16 and HM175/18f

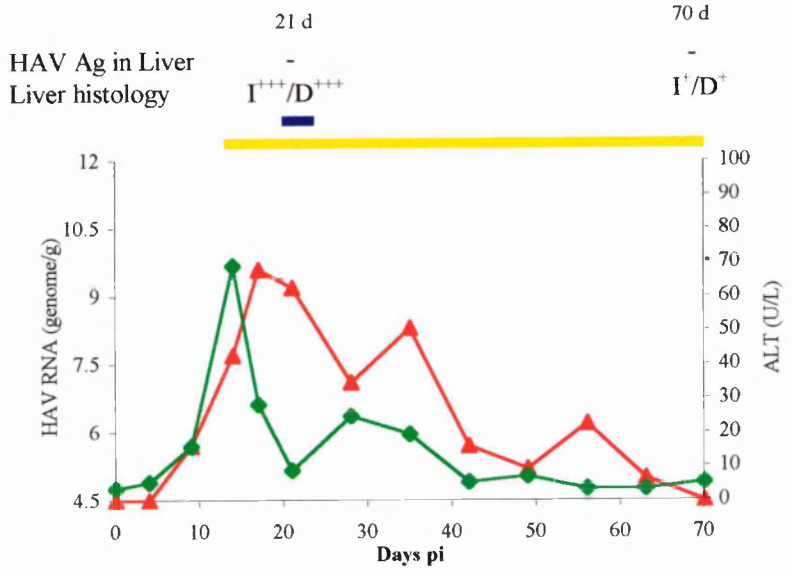
Residue	Wild type HM175	HM175 p16	HM175/18f
VP2 (54)	Lys	Arg	Arg
VP3 (91)	Thr	-	Lys
VP1 (271)	Ser	-	Pro



a) Tamarin C9

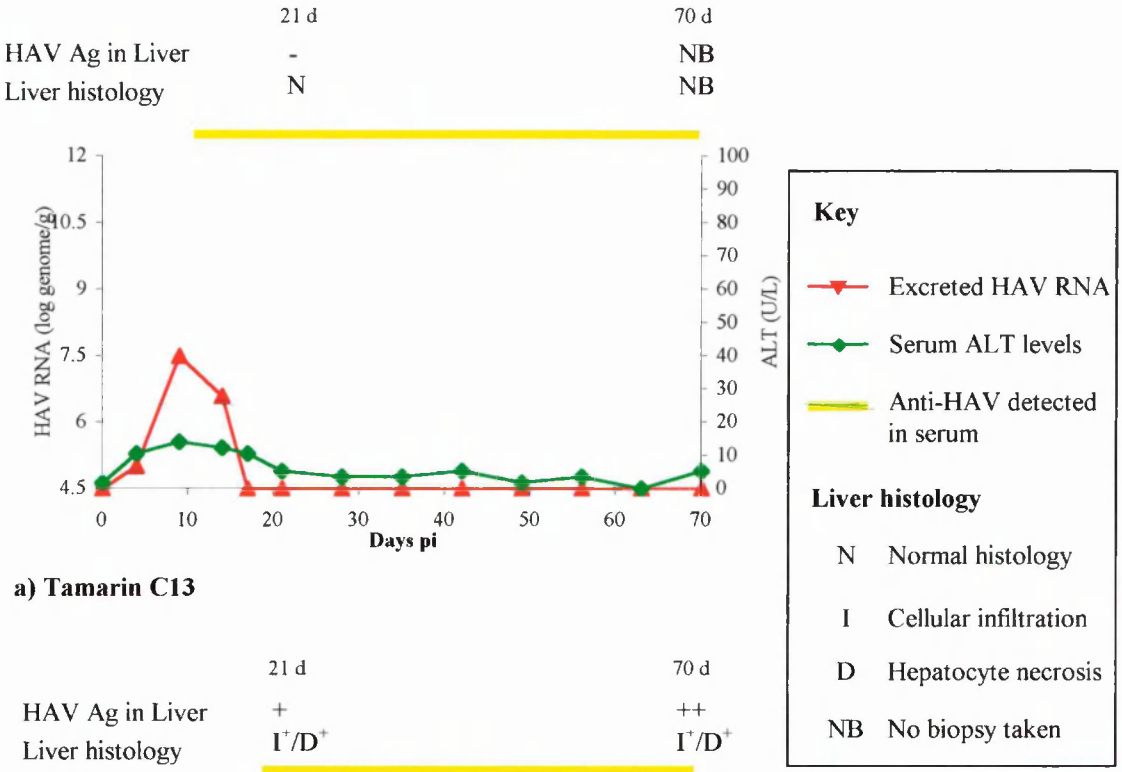


b) Tamarin C16

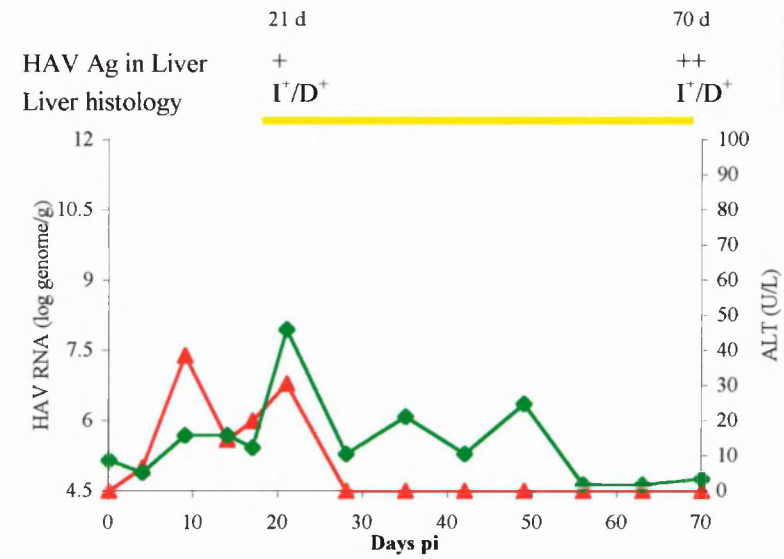


c) Tamarin C20

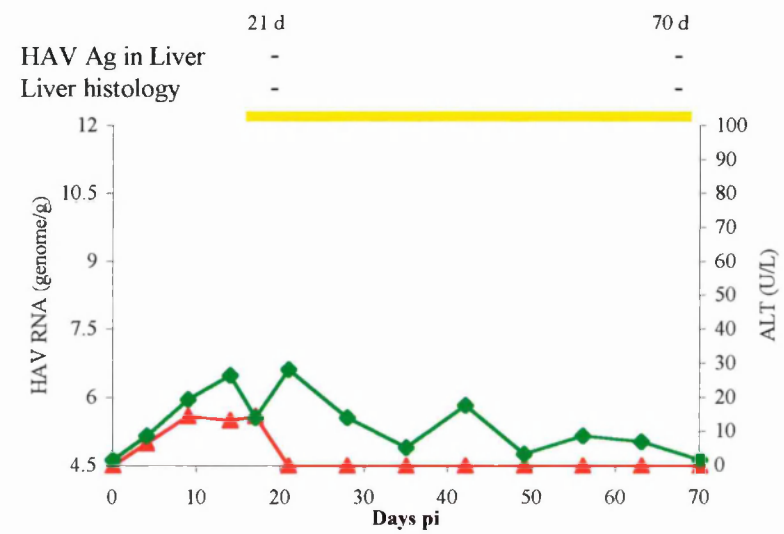
Figure 6.10 Replication and virulence of HAV HM175/wt in tamarins



a) Tamarin C13



b) Tamarin C17



c) Tamarin C19

Figure 6.11 Replication and virulence of HAV HM175/18f in tamarins

No faecal shedding was seen in animals inoculated with HAV HM175A.2. This suggests that the adaptations required for cytopathic growth of HAV in BS-C-1 cells differ from those which occur when the virus clonally isolated in FRhK-4 cells. The mutations in the BS-C-1 cell cytopathic variant appear to completely restrict the replication of the virus in the animal model whereas those occurring in HM175/18f cause a reduction in the titre and duration of faecal shedding of the virus.

Viraemia was only demonstrated in tamarins inoculated with the wild type HM175. Despite the fact that viraemia occurred in tamarin C16 infected with the wild type virus while levels of faecal shedding were comparable to that in the tamarins inoculated with HM175/18f, no viraemia was detected in the Group 2 tamarins. This may be due to the fact that the latter virus did not cause significant levels of liver damage as demonstrated by serum ALT levels and histological analysis of liver biopsies. The coincidence of viraemia with elevation of serum ALT in tamarins inoculated with the wild type virus also suggests that the presence of HAV in serum is due to leakage of the virus from damaged hepatocytes into the hepatic blood supply (Figure 6.10). A study of viraemia in human HAV infection showed detection of HAV in serum at the time of ALT elevation (Yotsuyanagi *et al.*, 1993).

The duration of viraemia was shorter than the 14-21 days reported by Lemon *et al.* (1990) using a tissue culture adapted strain of HAV which demonstrated a wild type phenotype in an owl monkey model. However, the sensitivity of the RIFA used to detect this virus was 1.3 log RFU ml^{-1} whereas the sensitivity of the RT-PCR assay used is 3.5 log genomes ml^{-1}. The occurrence of peak levels of viraemia ($\leq$ 4 log RFU ml^{-1}, 14-21 days p.i.) in the owl monkeys correlate well with the detection of HAV RNA in tamarins inoculated with HM175/wt in the present study. The duration of viraemia as detected by RT-PCR does not

appear to correlate with duration of virus excretion, time of seroconversion or peak serum ALT levels as may be expected. There may however be a correlation of the time of onset and duration of viraemia with the initial rate of increase and peak titre of viral shedding (Figure 6.10).

The mechanism by which liver damage occurs in individuals infected with HAV is unclear. Unlike other picornaviruses, there is little evidence that HAV is directly cytotoxic to host cells. Excretion of virus is usually at a maximum prior to the onset of the clinical symptoms associated with liver damage. The formation of immune complexes involving HAV capsid proteins, IgM and IgG and complement protein C3d does however coincide with the commencement of pathological changes in the liver (Margolis *et al.*, 1988). Cytotoxic antibodies could not be demonstrated in HAV infection (Slusracrzyk *et al.*, 1985; Gabriel *et al.*, 1986) whereas *in vitro* studies have implicated the involvement of the cell mediated immune response in the destruction of infected cells (Kurane *et al.*, 1985; Vallbracht *et al.*, 1986; Baba *et al.*, 1993). Despite appearance of antibodies to HAV while high levels of virus was being excreted from the liver, the extent of pathogenicity seen in liver biopsies taken from HM175/18f infected animals was much lower than that seen with the wild type. This tends to suggest that the cell mediated immune response upon inoculation with this cell culture adapted variant was less prolific. The absence of liver damage in animals inoculated with the HM175A.2 variant is probably due to the inability of this virus to infect and replicate to a detectable level in hepatocytes and therefore lack of antigen presentation to T lymphocytes. The levels of serum ALT detected in the three experimental groups of tamarins generally correlated with the severity of liver damage (Figures 6.10 and 6.11). However the absence of pathological changes in 2 of the tamarins in group 2 (inoculated with HM175/18f) which had raised serum ALT levels may be due the fact that the biopsy is only representative of a very small proportion of the whole liver.

Analysis of material from another area of the liver may have revealed the pathogenic changes typical of HAV infection. Thus the use of needle biopsy in studies of HAV virulence is limited due to the size of specimen obtained.

Replication of HAV in hepatocytes was demonstrated by immunohistochemistry. Again, the failure to demonstrate HAV antigen in 1 of the group 1 animals while virus was being excreted is probably due to the biopsy being taken from areas of the liver in which the virus was not replicating. Immunofluorescent staining of liver necropsies (Mathiesen *et al.*, 1980) revealed that only certain areas of the biopsy specimen contained HAV antigen. Viral antigen was only detected in the group 2 tamarin in which excretion of virus continued until the time of 21 day biopsy. However, a large area of viral antigen expression was also demonstrated in this animal at 70 days post inoculation when virus was no longer detected in the faeces suggesting that the virus had continued replicating in the liver at a level beneath the limits of detection by quantitative RT-PCR. In the same tamarin, elevated serum ALT levels continued to 49 days post inoculation compared with 35 days in tamarins inoculated with the wild type HM175. Small pockets of viral replication in the liver which cannot be detected in the faeces may be responsible for the protracted serum ALT elevations demonstrated in tamarins by Karayiannis *et al.* (1990). In that study however, a decline in antibody titre suggested that the virus was not actively replicating.

The attenuation of HAV HM175 p16, an African green monkey kidney cell adapted, non-cytopathic parent of HM175/18f, has been studied in owl monkeys (Taylor *et al.*, 1993). In the owl monkey model, only 1 out of 4 inoculated animals showed significantly raised serum ALT levels and low levels of viraemia. Faecal shedding was only detected in this animal. Seroconversion occurred in 3 of the 4 monkeys however, the mean time of

seroconversion was not until 103 days p.i.. All liver biopsies appeared normal and no HAV antigen was detected. Thus the HM175 variant p16 appears to be more attenuated in the owl monkey model than does HM175/18f in tamarins. These results may reflect variation in the degree of attenuation in different primates. Karron *et al.* (1988), showed differences in growth characteristics of wild type and tissue culture adapted variants of HAV HM175 in chimpanzees compared with marmosets. Alternatively, the additional mutations that occurred on further passage and clonal selection of HAV HM175/18f in FRhK-4 cells may have caused partial reversion to a wild type phenotype.

Tissue culture adapted strains of HAV are currently used in the production of formalin inactivated of HAV vaccines (Andre *et al.*, 1992; Werzberger *et al.*, 1992). The attenuation phenotype of some tissue culture adapted strains makes the production of live attenuated HAV vaccines a viable strategy. HAV strain HM175A.2 was able to elicit an antibody response at a similar time post infection as the more pathogenic strains studied. Quantatitive assay of the anti-HAV antibody produced in the inoculated tamarins is required to determine whether levels of antibody response to the different strains are comparable. HAV HM175 may prove useful in the development of an attenuated vaccine. However, much more work is required to show that the antibodies produced in response to this variant are protective and remain so over a long period of time. The stability of the attenuated phenotype would also need to be assessed in cell lines which have been approved for *in vitro* production of live attenuated vaccines such as MRC-5 or Vero cells. This study has highlighted the fact that culture of HAV in different cell lines leads to a variation in level of attenuation. If an adequate immune response is consistently produced using HM175A.2 in the absence of faecal shedding there will be little concern over the possibility of reversion to the virulent phenotype after inoculation. However, it is possible that there is a very low level of faecal shedding which is below the limit of detection of the

RT-PCR or cytopathic microtitre plate assays. Concentration of the material in a large volume faecal suspension by ultra centrifugation prior to analysis may clarify this point.

Chapter 7

GENERAL DISCUSSION

The initial aim of this project was to study the elimination and inactivation of HAV in blood products. Some viruses of major concern in blood virology are those for which there is no suitable cell culture based infectivity assay system, such as HCV, HBV, parvovirus B19 and wild type HAV. Therefore, the use of an alternative strategy for detection of these viruses, genome amplification, was investigated. The availability of tissue culture adapted strains of HAV also allowed the comparison of RT-PCR detection of the virus with assay of HAV infectivity in cell culture.

A relatively fast growing, tissue culture adapted strain of HAV (HM175A.2, Anderson, 1987) which produces cytopathic effects in B-SC-1 cells and achieves titres of up to 7 $\log_{10}$ TCIU ml^{-1} was used to develop a cytopathic microtitre plate assay. Statistical analysis of several assays showed that the repeatability of this assay was acceptable for routine use. Use of the cytopathic microtitre plate assay facilitated the quantitation of HM175A.2 in spiked factor VIII, tissue culture extracts and tamarin faecal samples. The technique could also be adapted to measuring neutralising antibody responses to HAV. A drawback of this assay is that it can only be used in the quantitation of viruses which have been adapted to cytopathic growth in cell culture. It may nevertheless be possible to further develop this assay so that non-cytopathic wild type or vaccine strains may be quantitated. In a competitive assay, the non-cytopathic HAV may be incubated with a stock antibody preparation at a standard dilution that will completely neutralise a given dilution of the cytopathic virus. Any virus in the sample would bind to the antibody and the remaining free antibody could then be assayed by the neutralisation assay described in Chapter 3 with cytopathic HAV as the challenge virus. Unfortunately time constraints did not permit the careful optimisation of virus and antibody dilutions required for the development of such an assay.

A genome amplification assay which was used to quantitate HAV RNA was also developed. Reverse transcription and PCR amplification of replicate serial dilutions made the measurement of RNA from non-cytopathic strains of HAV possible. The efficiency of this assay was assessed using *in vitro* transcribed RNA corresponding to the region of the genome that was amplified in the RT-PCR assay, which had also been quantitated by measurement of optical density. The titre of HAV RNA in samples could then be calculated in terms of genomes ml^{-1}.

The study monoclonal antibody affinity purification of HAV spiked factor VIII showed that the virus was not completely eliminated from the product during this process. HAV RNA was detected in the wash buffer and additional washing steps may help to further eliminate the virus. However, in order to guarantee the viral safety of factor VIII, additional elimination or inactivation steps are required.

Terminal dry heat inactivation of spiked HAV in lyophilised factor VIII was assessed in products of differing thermostabilities. At temperatures of 80 °C or above, the cell culture infectivity of the virus was quickly abolished, whereas the genome continued to be detected by RT-PCR until the factor VIII was heated to 100 °C for 8 hr. The use of temperatures as high as 100 °C is not suitable for all factor VIII preparations due to loss of product activity. The necessity of complete loss of PCR positivity is also questionable as the detection of viral RNA by this method does not indicate the presence of an infectious or even complete genome. While capture of the viral RNA at the 3 prime end followed by purification and RT-PCR amplification of the 5' non-coding region showed that the majority of genomes were intact after heat treatment,

assessment of the infectious nature of the heat treated genome is still required. An obvious approach to determine whether the heat treated RNA is infectious would be transfection of the genome into BS-C-1 cells. However, studies by van der Werf *et al.* (1986) and Sarnow (1989) showed that 1 μg of poliovirus RNA is required to produce 1.5-2 x 10^6 plaques in cell culture (approximately 10^5 poliovirus RNA molecules required for the formation of 1 plaque). Assuming similar titres of HAV RNA are required for transfection of BS-C-1 cells, a much higher concentration of the virus would be required in the spiked factor VIII in order to perform transfection studies.

Titres of HAV RNA measured using the quantitative RT-PCR assay were comparable to that of tissue culture infectious virus in the initial spiked factor VIII samples in both of the thermal inactivation experiments. In order to study further the relationship between infectivity and PCR positivity, a time course study of HAV replication in BS-C-1 cells was done. Levels of viral RNA in the medium and associated with the cells were compared with tissue culture infectious virus. The titres of HAV RNA in the medium throughout the time course were comparable to those of infectious virus with a ratio of 2 genome equivalents per tissue culture infectious unit. This figure is much lower than the ratio of 58 genome copies per RFU previously obtained by molecular hybridisation of a non-cytopathic strain of HAV (HM175 p16) and 2.4 x10^5 genome copies per RFU for wild type HAV HM175 (Jansen *et al.*, 1988). However, the ratio reported for the variant HM175 p16 (58:1) was determined after purification of the virus from lysed cells rather than from the supernatant. In this study, the ratio of infectious HAV HM175A.2 to HAV genomes was 19:1. The lower ratio may reflect the high degree of adaptation to growth in tissue culture attained by the variant HAV HM175A.2 over 30 passages of persistently infected cells followed by 8 virus passages

and 3 rounds of plaque purification (Anderson, 1987) compared with the 16 passages in cell culture of HAV HM175 p16.

Cell associated titres of HAV RNA were generally 1.3 log genomes ml^{-1} higher than that of infectious virus due to the presence of unencapsidated RNA, immature virus particles, virions containing defective HAV genomes or defective capsids containing functional RNA. Since the ratio of genome copies to infectious units determined for released virus appeared to be 2:1 in this study, only a small percentage of non-infectious virus particles were released. All samples were taken prior to the onset of visible cytopathic effect. Incubation of the cytopathic virus until cell death occurs may result in the release of the non-infectious viral RNA and thereby increase this ratio.

Particle to PFU ratios are usually determined by measurement of the number of particles of virus either by electron microscopy or optical density of a pure suspension of the virus and for picornaviruses, the ratios are usually between 1000 and 50 particles to PFU (Ruekert, 1990). However, the high titre of virus required for either of these methods has made this analysis difficult for HAV. Quantitative PCR could be used to determine the ratio of genome copies to PFU ratio of a well characterised virus such as poliovirus in order to elucidate the usefulness of such a ratio.

The titre of HAV RNA detected in a contaminated sample that has not been subjected to a viral inactivation protocol may be similar to that of infectious virus particles. However, these results are based on a ratio of RT-PCR determined genome equivalents to tissue culture infectivity and not the infectivity of the wild type virus *in vivo*. The

large number of susceptible animals required to properly determine the ratio of genome equivalents to *in vivo* wild type HAV infectivity would make such a study unfeasible.

The study of replication of HAV HM175A.2 in cell culture also demonstrated the growth characteristics of this virus in BS-C-1 cells. By quantitating the levels of RNA in terms of genome equivalents per ml, the rate of HAV RNA replication could be compared with the accumulation of infectious virus particles both associated with the cells and in the medium. The results showed a similar rate of uptake of this strain of HAV to that previously reported for poliovirus (Anderson *et al.*, 1987) with 90 % of the initial inoculum eclipsed after 1 hr. There was nevertheless a lag phase of 24 hr before levels of infectious virus began to rise. The cause of this long lag phase is unknown.

Comparison of levels of infectious virus with that of HAV RNA revealed that during the early stages of viral infection at 24 hr post infection, as much as 90 % of the genome may be encapsidated which may account for the slow growth of the virus and low yields obtained as the RNA is not available for further replication. The ability to quantitate the RNA so that it could be directly compared with infectious virus titres has therefore enabled the demonstration of the theory of Anderson and colleagues (1988) that the pool of RNA available for viral replication in the early stages of replication is low due to highly efficient encapsidation.

The quantitative RT-PCR method was also used to study the growth characteristics of HAV HM175/18f which is less cytopathic in BS-C-1 cells. The degree to which the RNA of this variant was taken up from the cell culture fluid before the release of progeny viral RNA was lower than that of HM175A.2 indicating a slower rate of attachment and subsequent uncoating the rate of HM175/18f. The rate of replication of

the HM175/18f RNA during the logarithmic growth phase was also slower than that of HM175A.2. The ability of HAV to cause cytopathology in BS-C-1 cells may be related to the rate of replication as suggested by Lemon *et al.* (1991) . In order to confirm this relationship, the rate of replication of HAV HM175/18f in FRhK-4 cells could be studied as this cell line exhibits greater cytopathology on infection with this strain than do BS-C-1 cells.

Zhang and colleagues (1995) showed that mutations in the region of wild type HAV RNA encoding the protein 2B and 2C resulted in a cytopathic rapidly replicating variant and further mutation in the P3 region, particularly 3A resulted in larger cytopathic plaque formation. The P2 proteins in which the mutations occur to produce cytopathic variants (2B and 2C) are both thought to function in transcription of the viral genome and interactions between these 2 proteins may occur during viral replication (Bienz *et al.*, 1990; Cho *et al.*, 1994; Zhang *et al.*, 1995). Since the cytopathology HM175/18f is specific to FRhK-4 cells, the fact that this virus does replicate in BS-C-1 cells tends to suggest that interactions of these proteins with host cell proteins may also be involved in the production of cytopathology. Upon passage in a cell line, the viral proteins may mutate in such a way as to interact more efficiently with the proteins specific to that cell line. Based on the function of the corresponding protein in poliovirus (Giachetti *et al.*, 1992) the 3A protein of HAV is thought to be involved in anchoring the genome in the endoplasmic reticulum by interaction with VPg and thereby aiding the synthesis of progeny RNA. The alteration of proteins involved in transcription may increase the rate of RNA replication resulting in cytopathology. Sequencing of the cytopathic variant HM175A.2 may help to further characterise the determinants involved in the production of cytopathology.

Whilst the HAV inactivation studies were performed using tissue culture adapted strains of the virus, it is the inactivation of the wild type virus in blood products that is essential. The adaptation of HAV to growth in tissue culture involves a number of mutations as discussed previously. These alterations in the genome of HAV often result in attenuation of virulence in the tissue culture adapted strains and may also give rise to altered characteristics during inactivation or elimination protocols. The degree of attenuation of the two tissue culture adapted strains used in this study (HM175A.2 and HM175/18f) was investigated. Tamarins, which are susceptible to infection with HAV, were inoculated with either the wild type HAV HM175 or the tissue culture adapted variants and the progress of disease monitored. The wild type HM175 replicated well in the animal model and viral RNA was excreted at high levels (up to 11 log genomes g^{-1}). Viraemia was also demonstrated in each of the tamarins infected with this virus. Histological analysis and measurement of serum ALT levels showed that liver damage occurred in each of these tamarins.

The variant HM175/18f also replicated in the animal model but at a lower level with a maximum of 6.8 log HAV genomes g^{-1} detected in the faeces. Subsequently, the degree liver pathology and elevation in ALT levels in the tamarins inoculated with this variant were lower than with the wild type. Viraemia was not detected in these tamarins. No replication or pathology in the animal was detected with the cytopathic virus HM175A.2. The adaptation to cytopathic growth in BS-C-1 cells therefore requires mutations in the HAV genome which also restrict the replication of the virus to growth *in vivo*, whereas only partial attenuation is seen upon adaptation to cytopathic growth in FRhK-4 cells. The use of HAV strains which cause both pathogenesis *in vivo* and cytopathogenicity in tissue culture may be beneficial in determining optimal elimination or inactivation procedures for blood products. The development of a

cytopathic microtitre plate assay for HM175/18f using FRhK-4 cells was not possible due to a lack of availability of viable cells. Such an assay would improve the suitability of the use of HM175/18f in further viral inactivation or elimination studies.

The detection of total anti-HAV responses in the inoculated animals appeared to be the same regardless of which HM175 variant used. The ability of HAV HM175A.2 to elicit an antibody response in tamarins with no pathogenic effects would make it a possible candidate strain for the production of a live attenuated vaccine. However of further study is required in order to determine the suitability of this strain for vaccine use. The immune response to HM175A.2 needs to more thoroughly characterised in terms of neutralisation ability. Time constraints did not permit assay of sera from inoculated animals in order to compare the levels of tissue culture neutralising antibody between experimental groups. Further long term studies are also required to determine the duration of neutralising antibody levels and the degree of protection achieved should be assessed by challenging animals inoculated with the attenuated strain with wild type virus. This study has shown that the adaptation of HAV to growth in different cell lines results in distinct characteristics *in vivo*. In the manufacture of vaccines, approved cell lines must be used such as Vero or MRC-5 cells. Therefore the virus would need to be carefully studied in a suitable cell line for adaptational mutations which may cause a reversion to the wild type phenotype.

The vaccination of patients such as haemophiliacs who receive large quantities of therapeutic medicines derived from human plasma has been suggested and implemented in some countries following the outbreaks of hepatitis A linked to blood products. In the short term, while alternative viral elimination or inactivation methods are sought, this option is satisfactory. However, the safety of blood products must be

assured with regard to other non-enveloped viruses such as parvovirus B19. HAV is an ideal virus for use in viral elimination and inactivation studies as tissue culture adapted strains can be quantitated both by gene amplification techniques and infectivity assay.

APPENDICIES

APPENDIX A

Comparison of nested PCR with the single PCR method in determining end point dilutions.

Introduction

The variability of amplicon band intensity in the PCR titrations suggested that not all cDNA containing samples may give a positive result. A low efficiency of visualisation by ethidium bromide stained, agarose gel electrophoresis may result in the failure to detect amplification products from those samples containing low copy number of cDNA. The apparent end point dilution would in this case be lower than the actual end point leading to an under estimation of the concentration of cDNA.

In order to determine whether ethidium bromide stained agarose gel electrophoresis of amplified DNA after conventional PCR was sufficient to visualise all positive samples, nested PCR was performed. In nested PCR, a small quantity of the product of conventional PCR is amplified using primers to an internal region of the amplicon. This method is not only useful in demonstrating specificity but also allows amplification of any PCR product which was not visible after the initial PCR to levels which can be clearly seen by ethidium bromide stained agarose gel electrophoresis.

Method

Samples: 1. HAV HM175A.2. Standard CS101/95.

2. HAV HM175/18f. Tamarin faecal sample (C13 d14, see Chapter 6)

RNA was extracted from the samples using the QIAGEN QIAmp kit and reverse transcribed using random primers as previously described. The resulting cDNA was diluted as follows.

Table 1. Dilutions of cDNA amplified by PCR

Dilution	HM175/18f (tamarin faecal sample C13d14)	HM175A.2 (PCR standard CS101/95)
1	1:10	$1:10^{1.5}$
2	$1:10^{1.5}$	$1:10^{2}$
3	$1:10^{2}$	$1:10^{2.5}$

Five replicates of each dilution of cDNA (5 µl per replicate), were amplified using primers A7a and A8a as described in chapter 2 (p 54-55). After 40 cycles of amplification, 1µl of each sample was added to 49 µl nested PCR mixes containing 1.5 mM magnesium chloride, 1.2 U Amplitaq Gold (Roche) and 20 pmoles each of primers N1 and N2. Amplitaq Gold was used in the nested PCR as the reaction had previously been optimised by Dr. Saldanha using this enzyme.

Nested Primers:
N1 (HAV nt 2754-2775) ACT GCT CTT GGA GCT GTC AGA T (forward)
N2 (HAV nt 3186-3166) CTT CCT GAG CAT ACT TGA GTC (reverse)

Amplification cycles:-

94 °C 2 minutes

94 °C 30 seconds
58 °C 20 seconds } 30 cycles
68 °C 30 seconds

68 °C 7 minutes

From the remaining first amplification mix and the amplified nested PCR mixes, 18 µl was run on an ethidium bromide stained 1 % agarose gel.

This comparison was repeated a total of 3 times for each sample.

Result

Figures 1 and 2 show that the use of nested PCR did not result in amplification of products of the first PCR which were not previously visualised by electrophoresis. The titres of RNA determined by both the single and nested PCR amplifications are shown in Tables 2 and 3 and the mean titres in table 4.

Table 2. Results of single PCR of the PCR standard CS101/95 (HM175 A.2) and the tamarin faecal sample C13d14 (HM175/18f).

		Number positive / Total number tested				Titre
Experiment		1:10	$1:10^{1.5}$	$1:10^{2}$	$1:10^{2.5}$	log genomes g^{-1}
HM175/18f	1	4/5	1/5	1/5	ND	5.55
	2	4/5	2/5	1/5	ND	5.64
	3	3/5	1/5	2/5	ND	5.59
						log genomes ml^{-1}
HM175A.2	1	ND	4/5	3/5	3/5	5.46
	2	ND	4/5	4/5	1/5	5.41
	3	ND	5/5	4/5	1/5	5.42

Table 3. Results of nested PCR of the PCR standard CS101/95 (HM175 A.2) and the tamarin faecal sample C13d14 (HM175/18f).

		Number positive / Total number tested				Titre
Experiment		1:10	$1:10^{1.5}$	$1:10^{2}$	$1:10^{2.5}$	log genomes g^{-1}
HM175/18f	1	4/5	1/5	1/5	ND	5.55
	2	4/5	2/5	1/5	ND	5.64
	3	3/5	1/5	2/5	ND	5.59
						log genomes ml^{-1}
HM175A.2	1	ND	4/5	3/5	3/5	5.46
	2	ND	4/5	4/5	1/5	5.41
	3	ND	5/5	4/5	1/5	5.42

Table 4. Mean titres obtained by single and nested PCR of the PCR standard CS101/95 (HM175 A.2) and the tamarin faecal sample C13d14 (HM175/18f).

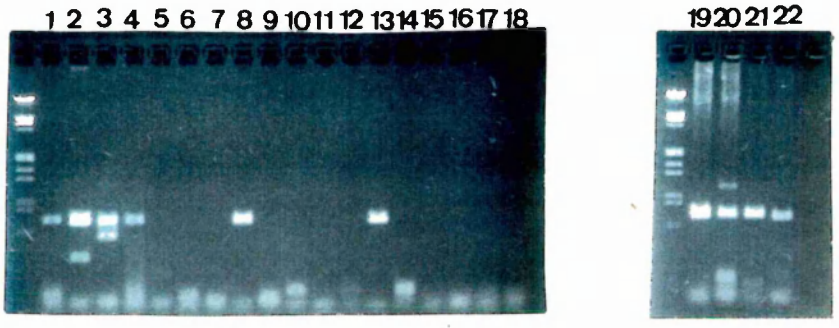
	Mean Titre	
	Single PCR	**Nested PCR**
HM175/18f (log genomes g^{-1})	5.59	5.59
HM175A.2 (log genomes ml^{-1})	5.43	5.43

Conclusion

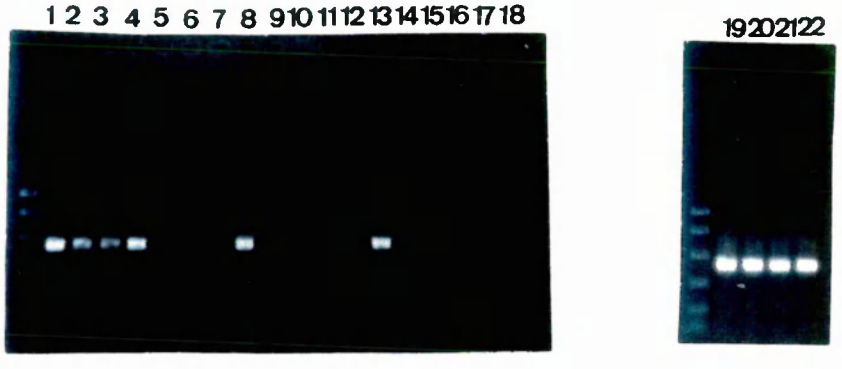
The titre of HAV HM175/18f RNA determined by both single round and nested PCR were the same as those obtained previously by single round PCR (5.6 log genomes / g). This indicates that the efficiency of the single round PCR was not altered in this study and that it is unlikely that the use of nested PCR would have improved the detection of HAV RNA in the more dilute samples. The efficiency of the single round PCR amplification was confirmed with the HM175A.2 PCR standard CS101/95.

Figure 1. Example nested PCR of the tamarin faecal sample C13d14 (HAV HM175/18f).

a. Single PCR amplification (Primers A7a and A8a)



b) Nested PCR (Primers N1 and N2)



Samples

1-5	cDNA diluted 1:10	18	Negative control water
6	Negative control water	19-20	Standard CS101 1:10
7-11	cDNA diluted $1:10^{1.5}$	21-22	Standard CS101 1:100
12	Negative control water		
13-17	cDNA diluted $1:10^{2}$		

Calculation of titre

Log titre of HAV RNA (log genomes ml^{-1}) = log titre (GLIM analysis) + log ($100/8.8^{a} \times 1000/3.5^{b}$) + 1^{c}

= 1.04 + 3.51 + 1

= 5.55 log genomes g^{-1}

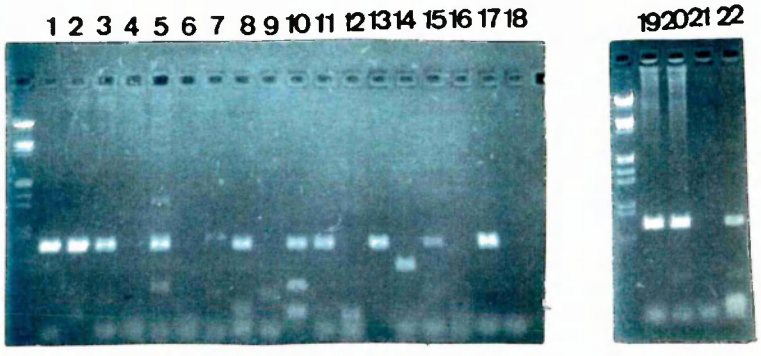
[a] Efficiency of reverse transcription (8.8%)

[b] Sample size (3.5 µl of a 10% suspension of the original sample in the reaction mix)

[c] Dilution of faeces (10% suspension)

Figure 2. Example nested PCR of the PCR standard CS101/95 (HAV HM175A.2).

a. Single PCR amplification (Primers A7a and A8a)



b) Nested PCR (Primers N1 and N2)

Samples

1-5	cDNA diluted $1:10^{1.5}$	18	Negative control water
6	Negative control water	19-20	Standard CS101 1:10
7-11	cDNA diluted $1:10^{2}$	21-22	Standard CS101 1:100
12	Negative control water		
13-17	cDNA diluted $1:10^{2.5}$		

Calculation of titre

Log titre HAV RNA (log genomes ml^{-1}) = log titre (GLIM analysis) + log (100/8.8[a] x 1000/3.5[b])

= 1.95 + 3.51

= 5.46 log genomes ml^{-1}

[a] Efficiency of reverse transcription (8.8%)

[b] Sample size (3.5 µl of the original sample in the reaction mix)

APPENDIX B - Reagents

General reagents

All chemicals were obtained from Sigma Chemical Co., Poole, UK unless otherwise stated

PBSA
171 mM sodium chloride
3.35 mM potassium chloride
10 mM disodium hydrogen orthophospate anhydrous
1.84 mM potassium dihydrogen orthophosphate

6 Salt PBS
140 mM sodium chloride
2.7 mM potassium chloride
20 mM disodium hydrogen orthophospate anhydrous
1.5 mM potassium dihydrogen orthophosphate
1 mM calcium chloride
0.5 mM magnesium chloride

Saline A
137 mM sodium chloride
5.37 mM potassium chloride
0.1 % glucose

Molecular cloning reagents

Bacterial cell culture media

	SOB	SOC	Luria-Bertani Broth
bactotryptone	2 %	2 %	1 %
yeast	0.5 %	0.5 %	0.5 %
sodium chloride	10 mM	10 mM	125 mM
potassium chloride	2.5mM	2.5 mM	-
magnesium chloride*	10 mM	10 mM	-
magnesium sulphate*	10 mM	10 mM	-
glucose**	-	20 mM	-

*1 M solution autoclaved separately and added to autoclaved SOC prior to use
**2 M solution filter sterilised and added to autoclaved SOC prior to use

AIX Luria agar plates

1.5 % (w / v)	Agar in Luria Bertani
100 $\mu g\ ml^{-1}$	Ampicillin
100 mM	IPTG
60 $\mu g\ ml^{-1}$	X-gal

Ethidium bromide stained agarose gels

1 x	TAE
1-2 %	electrophoresis grade agarose (Ultrapure, Life Technologies)
1 $\mu g\ ml^{-1}$	ethidium bromide

The agarose was dissolved in 1x TAE by heating and allowed to cool to 60 °C prior to the addition of ethidium bromide.

50 x TAE buffer

2 M	tris base
1 M	glacial ascetic acid
50 mM	ethylenediaminetetraacetic acid (EDTA)

10 x DNA loading buffer

50 %	glycerol (molecular biology grade)
5 x	TAE
0.1 %	bromophenol orange

The 50 % glycerol solution was incubated at 60 °C for 30 min prior to adding acridine orange

TE buffer pH 8.0

10 mM	tris HCl pH 8.0
1 mM	ethylenediaminetetraacetic acid (EDTA)

FSB

10 mM	potassium acetate
100 mM	potassium chloride
45 mM	manganese cloride
10 mM	calcium chloride
3 mM	colbalt chloride
10 %	glycerol

Molecular weight markers

DNA

1 DNA molecular weight marker III (Boehringer Mannheim, Lewes, UK)
λ-DNA cleaved with EcoR I and Hind III
13 fragments 0.12-21.2 kbp

125, 564, 831, 947, 1375, 1584, 1904, 2027, 3530, 4268, 4973, 5148, 21226 bp

2 PCR markers (Promega Corp., Southampton, UK)
6 fragments 10-1000 bp

50, 150, 300, 500, 750, 1000 bp

RNA markers (Promega Corp., Southampton, UK)
9 RNA fragments 0.28-6.58 kb

281, 623, 955, 1383, 1908, 2604, 3638, 4981, 6583 b

PCR primers (5 prime non-coding region)

	57	77
HA1F	5' -GAC-TTG-ATA-CCT-CAC-CGC-CGT-	3'
	312	292
HA1R	5' -AGA-CTC-CTA-CAG-CTC-CAT-GCT-	3'

APPENDIX C - Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BCIP	bromochloroindolylphosphate
BMEM	buffered Eagles Minimum Essential Medium
BPL	Bio Products Laboratories
cDNA	complementary strand DNA (produced by reverse transcription)
CNS	central nervous system
CPE	cytopathic effect
CV	coefficient of variation
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dNTPs	deoxynucleoside triphosophates
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacitic acid
FCS	foetal calf serum
GE	genome equivalents
HAV	hepatitis A virus
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
ICD	Isocitrate dehydrogenase
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IMS	industrial methylated spirit
IRES	internal ribosomal entry site
ISG	immune serum globulin
LD	lactate dehydrogenase
MEM	Eagles Minimum Essential Medium
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NBT	nitro blue tetrazolamine
NIBSC	National Institute for Biological Standards and Control
PBSA	phosphate buffered saline A
PCR	polymerase chain reaction
PFU	plaque forming units
RFU	radioimmunofocus forming unit
RIA	radioimmunoassay
RIFA	radioimmunofocus assay
RIFIT	radioimmunofocus inhibition test
RNA	ribonucleic acid

RNAse	ribonuclease
RSV	respiratory syncytial virus
RT-PCR	reverse transcription and PCR
SDS	sodium dodecyl sulphate
SNBTS	Scottish National Blood Transfusion Service
SSC	saline sodium citrate buffer
$TCID_{50}$	50% tissue culture infectious dose
TCIU	tissue culture infectious unit
TNF	tumour necrosis factor
UTR	untranslated region
UV	ultraviolet
WHO	World Health Organisation

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